CURRENT PROTOCOLS IN PROTEIN SCIENCE

VOLUME 2

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CHAPTER 11
Chemical Analysis

INTRODUCTION

Chapter 11 is devoted to methodologies related to determining the primary structure of proteins. Although the techniques detailed can be applied to proteins available in large amounts, particular emphasis is placed on analyzing proteins available in limited (picomole) quantities. Amino acid analysis, described in *UNIT 11.9*, is one of the best methods to quantify peptides and proteins. It also serves as a valuable analytical method for evaluating synthetic peptide or recombinant protein purity, identifying proteins based on composition, and detecting odd amino acids. Primary sequence analyses (*UNIT 11.10*) are used to identify proteins and to obtain information that can be used to design oligonucleotide probes that can be used to clone the gene encoding the protein of interest. Also, with the proviso that contaminating proteins may not be sequenceable, and hence undetectable due to blocked N termini, some assessment of purity can be made from N-terminal sequence analyses.

The usual approach for obtaining primary sequence analysis is to attempt to sequence the N terminus of the intact protein; however, for one reason or another, proteins may lack a free N-terminal amino group (i.e., they are blocked) and are consequently refractory to Edman degradation. One common N-terminal blocking group, pyrrolidone carboxylic acid, formed by cyclization of glutamine or glutamic acid, can often be removed by treatment with the enzyme pyroglutamate amino peptidase, as described in *UNIT 11.7*. This unit also describes methods for obtaining N-terminal sequence of N^{α}-acyl-proteins, but in this case, the protein must first be fragmented. If purified, blocked protein cannot be "unblocked" by these or other means, it is usually necessary to fragment the protein by enzymatic and/or chemical methods (*UNITS 11.1 & 11.4*, respectively) and to isolate individual protein fragments by reversed-phase high performance liquid chromatography (RP-HPLC; *UNIT 11.6*) or other means in order to obtain primary sequence data. Even if the N terminus of a protein is not blocked, such an approach frequently constitutes the most economical use of protein, in that much more sequence can often be obtained from a limited amount of protein using this approach than if just the N terminus were sequenced.

Carboxy-terminal sequence analysis detailed in *UNIT 11.8* is used for direct confirmation of the C-terminal sequence of native and expressed proteins, for identifying post-translational proteolytic cleavages, and for obtaining partial sequence information in order to design oligonucleotide probes for gene cloning. *UNIT 11.8* contains an automated chemical method and a manual method based on carboxypeptidase digestion for obtaining such information. The enzymatic protocol requires amino acid analysis capabilities as detailed in *UNIT 3.2* and *UNIT 11.9*.

Because the final step in protein purification is usually SDS-PAGE, electroblotting to solid supports such as polyvinylidene difluoride (PVDF) membrane is the simplest and most common procedure for recovering proteins in high yield and free of contaminants such as SDS. However, because elution of proteins from such solid supports is often very inefficient, procedures for enzymatic and chemical digestion of proteins on solid supports have been developed (*UNITS 11.2 & 11.5*, respectively). In some cases protein losses are incurred during transfer from the SDS-polyacrylamide gel to the solid support, or digestion on the solid support may be particularly inefficient. For such cases, special

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enzymatic digestion protocols have been developed to digest proteins directly in the resolving gels (*UNIT 11.3*). These protocols are tailored to deal with the high concentrations of SDS present in such gels, which normally inhibits enzymatic hydrolysis. A support protocol provides a method for determining sample concentration by amino acid analysis.

As described in *UNIT 11.6*, RP-HPLC is a fundamental tool for the isolation and analysis of peptides generated by various cleavage procedures. Peptides are separated on a hydrophobic stationary phase and eluted with a gradient of increasing organic solvent concentration. Depending on the amount of peptide, different-sized columns are recommended—e.g., narrow-bore, microbore, and capillary; protocols for each column type are provided. A support protocol is given detailing the construction of a capillary HPLC system, as they are not as yet commercially available. In order to assess the purity of the peptides present in the HPLC fractions, protocols for performing matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and capillary electrophoresis (also see *UNIT 10.9*) analyses are included.

In the end, data obtained through these procedures can be used to confirm the identity of a known protein, correlate a protein with a known gene sequence, or form the basis for the synthesis of oligodeoxynucleotides (primers or probes) to clone a gene corresponding to the protein sequence.

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Enzymatic Digestion of Proteins in Solution

Analysis of protein covalent structure is less complex and more accurate when performed on peptides derived from the larger protein. In contrast to acid-promoted total hydrolysis (e.g., in 6 N HCl), peptides are typically generated by selective proteolysis—i.e., by specifically cleaving peptide bonds with endoproteases that have varying degrees of specificity.

The basic protocol can be used to generate peptide fragments from intact, undenatured proteins. Fragments can be analyzed directly by mass spectrometry (MS) or, more often, are first separated by reversed-phase HPLC (RP-HPLC) and then analyzed by MS or automated sequencing. Expertise with these analytical and chromatographic techniques is required, or else the procedures must be performed in association with a skilled operator.

Most proteins are resistant to enzymatic proteolysis under nondenaturing conditions (see Table 11.1.2) or are not soluble in aqueous solution. Digestion procedures performed in the presence of chaotropic agents and SDS are described in Alternate Protocols 1 and 2. Support Protocols 1 and 2 provide instructions for preparing enzyme stocks and reducing and alkylating peptides prior to sequencing or HPLC analysis.

DIGESTION OF PROTEINS UNDER NONDENATURING CONDITIONS

BASIC PROTOCOL

Protein is solubilized in an appropriate digestion buffer and subjected to enzymatic proteolysis. Fragmentation is monitored by SDS-PAGE or RP-HPLC, followed by preparative RP-HPLC of the completed digest. The procedure is recommended for checking protease sensitivity of unknown proteins or for preparing proteolytic fragments that can be sequenced when working with proteins already shown to be sensitive to a particular protease. The minimal amount of protein required is 100 pmol.

Materials

100 pmol to 5 nmol protein sample, as pellet or solution
1 × and 10× digestion buffer (see Table 11.1.1)
20% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 20% octyl glucoside, or 20% Nonidet P-40 (NP-40; Calbiochem)
100% acetonitrile
1 µg/µl enzyme stock (see Support Protocol 1; store at –20°C)
Trifluoroacetic acid (TFA; Pierce)
Solvent A: 0.1% (v/v) TFA in water
Solvent B: 0.09% (v/v) TFA in 70% (v/v) acetonitrile (Burdick & Jackson)

Sonicator bath (Branson)
Phast Gel system (Pharmacia), optional
HPLC system, with C18 or C4 reversed-phase column, 4.6-mm or 2.1-mm (e.g., Vydac);UV detector; chart recorder

Additional reagents and materials for SDS-PAGE (*UNIT 10.1*), gel staining (*UNIT 10.5*), and HPLC analysis of peptides (*UNIT 11.6*)

1. Dissolve protein pellet in minimal volume of appropriate 1× digestion buffer. If protein sample is already in solution, add 0.1 vol of 10× digestion buffer.

   Final protein concentration must be >1 µg/20 µl. If sample is too dilute and volume is over 0.5 ml, use Centricon microconcentrator (see manufacturer's instructions) to reduce the volume.

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Table 11.1.1 Conditions for Endoprotease Activity[a,b]

Condition	Enzyme									
	T	C	KC	DN	EC	S	H	P	E	PA
Nondenaturing										
Buffer	0.1 M AB	0.1 M AB	0.1 M AB	0.1 M AB	0.05 M AB	0.1 M AB	0.1 M AB/ 1 mM $CaCl_2$	0.1% TFA	0.2 M TC	0.1 M AB/ 1 mM EDTA
Time	2 hr	2 hr	2 hr	5 hr	5 hr	1 hr	2 hr	1 hr	18 hr	5 hr
Low/high pH										
Acid	—	—	—	—	—	—	—	+	—	—
NH_4OH	—	—	0.1 M	—	—	1 M	—	—	—	—
Chaotropes										
Urea	4 M	2 M	8 M	2 M	2 M[c]	8 M[c]	8 M[c]		2 M	8 M[c]
Buffer	0.1 M AB/ +5 mM $CaCl_2$	0.1 M AB	0.2 M TC	0.1 M AB	0.05 M AB	0.1 M TC	0.1 M AB/ +5 mM Ca 0.1 M TC/ +5 mM Ca	ND	0.2 M TC	0.1 M AB/ 1 mM EDTA
Time	15 hr	5 hr	5 hr	5 hr	5 hr	1 hr	2 hr		18 hr	5 hr
Gu·HCl	—	2 M	2 M	1 M[c]	1 M[c]	2 M[c]	—	2 M	2 M[c]	2 M
Buffer		0.2 M AB	0.2 M TC	0.1 M AB 0.2 M TC	0.05 M AB	0.1 M AB		12 mM HCl	0.2 M TC	0.1 M AB/ 1 mM EDTA
Time		24 hr	5 hr	18 hr	18 hr	2 hr		2 hr	18 hr	18 hr
Detergents[d]										
SDS	<0.1%	0.1%[c]	1%	<0.1%	0.1%[c]	1%	0.1%[c]	ND	0.1%	—
CHAPS	2%	1%	2%	2%	2%[c]	2%[c]	2%	ND	1%	1%
OG and NP-40	2%	1%	2%	2%	2%[c]	2%[c]	2%	ND	1%	2%[c]
Organic solvents[d]										
MeCN	40%	30%	40%	40%	20%[c]	40%	40%	20%[c]	40%	40%
IPA	40%	20%	40%	40%	20%[c]	40%[c]	40%[c]	20%[c]	20%	40%
Reducing agent										
2-ME	—	—	0.5%	0.1%	0.5%	ND	ND	ND	ND	ND

[a]Buffers listed here are 1× stocks; it may be necessary to prepare 10× stocks for certain parts of the protocols. Listed here are the highest concentrations of additives that still permit adequate proteolysis.

[b]Abbreviations: Enzymes: C, chymotrypsin; DN, endoproteinase Asp-N; E, elastase; EC, endoproteinase Glu-C; H, thermolysin; KC, endoproteinase Lys-C; P, pepsin; PA, papain; S, subtilisin; T, trypsin. Other: —, does not work; +, works at pH 2.0; AB, ammonium bicarbonate; Gu·HCl, guanidine hydrochloride; IPA, isopropanol; 2-ME, 2-mercaptoethanol; MeCN, acetonitrile; OG, octyl glucoside; TC, Tris·Cl, pH 8.5; TFA, trifluoroacetic acid.

[c]Restricted digest. Note that for the 8M urea conditions, the final concentration is actually 7.3 M after addition of the buffer.

[d]Concentrations higher than 2% CHAPS, octyl glucoside, and NP-40, and 40% acetonitrile and isopropanol were not tested.

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2. Incubate sample 5 min at 37°C, then sonicate 1 min. Repeat until pellet is completely dissolved. If pellet does not dissolve, add CHAPS (2% final), octyl glucoside (2% final), NP-40 (2% final), or acetonitrile (20% to 40% final; Table 11.1.1) to digestion solution.

   These solubilization agents do not interfere with digestion (see Table 11.1.1).

   NOTE: *Digestion solution should not contain protease inhibitors. All proteases are inhibited by leupeptin. In addition, trypsin, chymotrypsin, endoproteinase Lys-C, endoproteinase Glu-C, subtilisin, and elastase are inhibited by phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), and aprotinin; thermolysin and endoproteinase Asp-N are inhibited by EDTA.*

3. Transfer 1 µl sample to pH indicator strip. If pH is not correct, add sufficient 10× digestion buffer to increase buffer strength by 100 mM (see Table 11.1.1) and recheck the pH.

   For pepsin, the pH must be 2.0. Subtilisin and endoproteinase Lys-C work well in a pH range of 7 to 10.5. For all other proteases, pH should be 8.0 to 8.5.

4. Add 1 µg/µl enzyme stock solution to a final enzyme/substrate ratio of 1:50 to 1:10 (w/w).

   Final enzyme concentration should be >1 µg/100 µl.

5. Incubate at 37°C for the amount of time recommended for the selected protease (see Table 11.1.1).

6. Determine extent of digestion by analyzing 0.5 to 1 µg digest by SDS-PAGE (e.g., using a Phast Gel). Stain with Coomassie brilliant blue (*UNIT 10.5*). Alternatively, analyze 25 to 500 pmol by RP-HPLC (use a 2.1-mm column for 25 to 200 pmol protein or a 4.6-mm column for 200 to 500 pmol protein). Keep remaining sample on ice.

   The procedure should not consume >10% of the sample. Phast Gels are useful when protein amounts are limited.

7. If sample is digested, proceed to preparative RP-HPLC (step 8). If sample is not digested, check pH and adjust if necessary. Add a second aliquot of enzyme and incubate at 37°C for an additional 30 min to full incubation time. Check extent of digestion as in step 6.

   If sample is still not digested after incubation with a second aliquot of enzyme, try a different enzyme. If the second enzyme is ineffective, denature the substrate (see Alternate Protocol 1 and Alternate Protocol 2).

   If HPLC analysis cannot be performed immediately, TFA should be added to a final concentration of 2% and the sample should be stored at –70°C. Digestion samples may be kept indefinitely under these conditions.

8. Using a clean Hamilton syringe, inject remaining digested sample onto column. Program HPLC to run isocratically at 5% solvent B until baseline returns to its original position. Set flow rate to 100 µl/min for a 2.1-mm column or 1 ml/min for a 4.6-mm column. Run gradient as follows:

   5% to 50% solvent B in 45 min;
   50% to 100% solvent B in 25 min.

   A 4.6-mm column can easily accommodate 1 to 2 ml sample volume. If the sample contains organic solvent, it should be diluted five-fold with solvent A.

   See UNIT 11.6 for further details on HPLC separation of peptides.

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ALTERNATE PROTOCOL 1

DIGESTION OF PROTEINS IN THE PRESENCE OF UREA OR GUANIDINE·HCl

Because many proteins are resistant to protease digestion in their native state, the majority of digests are carried out in the presence of urea and guanidine·HCl. The highest concentrations of these chaotropes compatible with the activity of the most commonly used proteases are listed in Table 11.1.1. Including chaotropes in the digest mixture avoids having to denature (or reduce and alkylate) substrate proteins prior to digestion. This, in turn, eliminates the need to remove the denaturants by chromatography or dialysis before adding the protease, a step which can cause heavy losses of substrate proteins present in subnanomole quantities.

Proteins are solubilized and denatured in high concentrations of urea or guanidine·HCl, and the denaturant is then diluted to a concentration high enough to prevent refolding of the substrate but low enough to allow protease digestion. Fragmentation is monitored by RP-HPLC. The procedure has a high success rate and is most often used as the method of first choice. The protocol requires ≥200 pmol of protein.

Additional Materials (also see Basic Protocol)

8 M urea (sequenal grade, Pierce; store up to several weeks at room temperature over Bio-Rad AG 501-X8 mixed-bed resin)
6 M guanidine·HCl (sequenal grade, Pierce), prepared fresh immediately before use
Milli-Q water or equivalent
50°C water bath

1a. *If sample is solid:* Dissolve protein pellet in a minimal vol of 8 M urea or 6 M guanidine·HCl. Heat sample 5 min at 37°C, then sonicate 1 min. Repeat heating and sonication until pellet is dissolved.

1b. *If sample is already in solution and precipitation is not desirable:* Add solid guanidine·HCl to give a final concentration of 6 M: i.e., add 104 mg guanidine·HCl per 100 µl sample volume (final volume will then be 182 µl).

2. Heat sample 30 min at 37°C (for 8 M urea) or 30 min at 50°C (for 6 M guanidine·HCl).

3. Allow sample to cool to room temperature. Add water and appropriate 10× digestion buffer to achieve desired final concentration of chaotropic agent as follows:

For 8 M urea: undiluted, add 0.1 vol 10× buffer
For 4 M urea: 0.8 vol water and 0.2 vol 10× buffer
For 2 M urea: 2.6 vol water and 0.4 vol 10× buffer
For 2 M guanidine·HCl: 1.7 vol water and 0.3 vol 10× buffer.

Final substrate concentration should be >1 µg/10 µl; e.g., for a fourfold dilution (e.g., 8 M to 2 M urea), the initial protein concentration (before denaturation) should be >1 µg/2.5 µl. If the sample is too dilute, use a Centricon microconcentrator (see manufacturer's instructions) to reduce the volume.

For the 8 M urea conditions, after addition of the buffer the final urea concentration is actually 7.3 M.

4. Transfer 1 µl sample to pH indicator strip. If pH is not correct, add sufficient 10× digestion buffer to increase buffer strength by 100 mM and recheck the pH.

For pepsin, the pH must be 2.0. Subtilisin and endo Lys-C work well in a pH range of 7.0 to 10.5. For all other proteases, pH should be 8.0 to 8.5. Guanidine·HCl requires double buffer strength to adjust to mild alkaline pH.

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5. Add 1 µg/µl enzyme stock to a final enzyme/substrate ratio of 1:25 to 1:10 (w/w). Vortex sample lightly to mix. Microcentrifuge 10 sec at high speed.

   Enzyme concentration should be >1 µg/50 µl.

   Autolysis of proteases occurs in 8 M urea and in 2 M guanidine·HCl. The resulting peaks (run an auto-digestion control in parallel) must be accounted for when sample digest is analyzed by RP-HPLC.

6. Incubate sample at 37°C for the amount of time recommended for the selected protease (see Table 11.1.1).

7. Determine extent of digestion by analytical RP-HPLC (see Basic Protocol step 6). Store remainder of sample on ice.

8. If digestion is complete, proceed to step 9. If digestion is incomplete, check and adjust pH, then redigest (see Basic Protocol, step 7).

   If more enzyme is added to continue digestion, enzyme/substrate ratio must be <1:10. Do not use SDS-PAGE to check digests performed in the presence of guanidine·HCl, as guanidine·DS salts are insoluble. The analytical procedure should not consume >10% of the sample.

   If HPLC analysis cannot be performed immediately, samples may be stored indefinitely at –70°C.

9. If desired, reduce and alkylate peptides (see Support Protocol 2).

   NOTE: *Samples alkylated with 4-vinylpyridine (Support Protocol 2) must be injected onto an HPLC column immediately. They cannot be stored even for 1 hr! On a 2.1–mm microbore column, operated at a 100 µl/min flow, it may take up to an hour for the 2-ME and 4-vinylpyridine to wash off the column.*

10. Separate peptides by RP-HPLC (see Basic Protocol, step 8).

DIGESTION OF PROTEINS IN THE PRESENCE OF SDS

ALTERNATE PROTOCOL 2

Proteins must be in solution before they can be adequately digested. Adding SDS to a protein sample is an excellent way to keep hydrophobic proteins (e.g., membrane proteins) in aqueous solution. At concentrations of ≥1%, and at elevated temperatures, SDS will also efficiently denature the substrates. The highest SDS concentrations compatible with the most commonly used proteases are listed in Table 11.1.1.

Protein is solubilized (and denatured, if necessary) in SDS and digested with the desired protease. The extent of digestion can easily be monitored by SDS-PAGE. SDS is precipitated as the guanidine·DS salt and removed by centrifugation prior to separating the digested peptides by RP-HPLC. SDS can also be used to generate larger peptide fragments by providing conditions favorable for limited protease digestion; the fragments can then be separated by SDS-PAGE, followed by electroblotting onto a membrane, if desired.

Additional Materials (also see Basic Protocol)

1× digestion buffer with 1% or 0.1% (w/v) SDS (see Table 11.1.1)
10% and 1% (w/v) SDS (Bio-Rad)
10× digestion buffer (no SDS)
1 M guanidine·HCl (sequenal grade, Pierce), prepared immediately before use
60°C and 95°C water baths

1a. *If sample is solid:* Dissolve protein sample in a minimal volume of digestion buffer containing 1% (or 0.1%) SDS. Heat sample 5 min at 37°C and sonicate 1 min. If

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pellet does not dissolve, heat sample 5 min at 60°C and sonicate 1 min. Repeat heating and sonication until protein dissolves.

1b. *If sample is already in solution:* Add 0.1 vol of 10% or 1% SDS (1% or 0.1% final) and 0.1 vol of 10× digestion buffer.

Buffer composition and optimal SDS concentration differ for each protease. See Table 11.1.1 for recommended buffer conditions.

IMPORTANT NOTE: *Solution should not contain guanidine·HCl, as guanidine·DS salt will precipitate. Final substrate concentration should be >1 µg/10 µl. If protein sample is too dilute, use a Centricon concentrator (see manufacturer's instructions) to reduce the sample volume.*

2. Transfer 1 µl sample to pH indicator strip. If pH is not correct, add sufficient 10× digestion buffer to increase buffer strength by 100 mM and recheck pH.

For pepsin, pH must be 2.0. Subtilisin and endoproteinase Lys-C work well in a pH range of 7.0 to 10.5. For all other proteases, pH should be 8.0 to 8.5.

3. If nondenatured protein is protease resistant, heat sample 5 min at 95°C to denature protein. Allow sample to cool to room temperature.

For a partial list of protease-resistant proteins, see Table 11.1.2.

4. Add 1 µg/µl enzyme stock to a final enzyme/substrate ratio of 1:25 to 1:10 (or 1:100 to 1:50 for a limited digestion). Vortex sample lightly to mix. Microcentrifuge sample 10 sec at high speed.

Enzyme concentration should be >1 µg/50 µl (or less—e.g., 1 µg/200 µl—for a limited digestion).

Table 11.1.2 Results of Proteolysis of Undenatured Substrates[a,b,c]

Substrate	#AA	Intrachain disulfide bonds	T	C	KC	DN	EC	S	H	P
Cytochrome *c*	104	0	+++	+++	+++	+++	–	+++	+++	+++
Triosephosphate isomerase	248	0	–	–	–	–	–	–	–	–
Carbonic anhydrase	259	0	–	–	+++	+	+	+++	–	+++
Trypsin inhibitor	56	3	–	–	–	–	–	–	–	–
RNase	124	3	–	–	–	–	–	+++	–	++
Lysozyme	129	4	–	–	–	–	–	+++	–	–
Superoxide dismutase	151	1	–	–	–	–	–	–	–	–
β-Lactoglobulin	162	2	+++	+++	+++	+++	+++	+++	+++	–
Ovalbumin	385	1	–	–	–	–	–	+++	–	–
Bovine serum albumin	577	17	+++	–	+++	+	–	+++	–	+++

[a]Digests were carried out at 37°C with enzyme/substrate ratios of 1:25 (w/w); buffer compositions and incubation times were as listed in Table 11.1.1 (top rows) except for subtilisin digest of lysozyme, which was done for 5 hr. Analysis was done by RP-HPLC.

[b]Abbreviations: #AA, length of protein in amino acids; C, chymotrypsin; DN, *Pseudomonas fragi* endoprotease Asp-N; EC, *Staphylococcus aureus* V8 endoprotease Glu-C; H, thermolysin; KC, *Achromobacter* protease I, or endoprotease Lys-C; P, pepsin; S, subtilisin; and T, trypsin.

[c]Results: +++, complete digest; ++, incomplete digest; +, very incomplete digest (>50% substrate left); –, no digest (no peptides, only substrate).

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5. Incubate at 37°C for the amount of time recommended for the selected protease (see Table 11.1.1). For a limited digest, incubate 15 to 30 min at 37°C.

6. Determine extent of digestion by analyzing 0.5 to 1 µg sample by SDS-PAGE (e.g., using a Phast Gel). Stain sample with Coomassie brilliant blue (*UNIT 10.5*).

   Phast Gels are useful when protein amounts are limited.

7. If sample is fully digested, proceed to step 8. If sample is not fully digested, check and adjust pH, then redigest (see Basic Protocol, step 7). If a limited digest is desired, proceed to step 8.

   If more enzyme is added to achieve a more extensive digest, the enzyme/substrate ratio must be <1:10. The analytical procedure should not consume more than 10% of the sample. Do not use RP-HPLC to check digest, because SDS and RP-HPLC are not compatible.

8. To remove SDS, add 1 vol of 1 M guanidine·HCl to sample and tap tube lightly to mix.

   A white precipitate will form immediately.

9. Microcentrifuge sample 10 min at high speed. Transfer supernatant to clean microcentrifuge tube or inject directly onto RP-HPLC.

   If HPLC analysis cannot be performed immediately, sample may be stored indefinitely at –70°C.

10. Reduce and alkylate sample if desired (see Support Protocol 2)

11. Separate peptides by RP-HPLC (see Basic Protocol, step 8).

    Do not inject solids onto HPLC column.

PREPARING AND USING ENZYME STOCKS

SUPPORT PROTOCOL 1

This protocol details the handling of proteases—the solubilization of the enzyme from manufacturer stocks, the adjusting of concentrations and testing of activities under the most stringent conditions (listed in Table 11.1.1), and the thawing of enzyme stocks prior to experimental use.

Materials

Enzymes:
- Trypsin (sequencing grade, Boehringer Mannheim 1047-841 or Promega V5111)
- Chymotrypsin (sequencing grade, Boehringer Mannheim 1334-131)
- Endoproteinase Glu-C (endo Glu-C; sequencing grade, Boehringer Mannheim 1047-817)
- Endoproteinase Asp-N (endo Asp-N; sequencing grade, Boehringer Mannheim 1054-859)
- Endoproteinase Lys-C (endo Lys-C; Wako Chemicals 129-02541)
- Subtilisin (Sigma P5380)
- Thermolysin (Sigma P1512)
- Pepsin (Sigma P6887)
- Elastase (Sigma PE0258)
- Papain (Sigma P3125)

Milli-Q water or equivalent
Trifluoroacetic acid (TFA; Pierce)
5 mg/ml cytochrome *c* (Sigma)
10× digestion buffer (see Table 11.1.1)

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5 mg/ml β-lactoglobulin (Sigma)
5 mg/ml triosephosphate isomerase (Sigma)
8 M urea (sequenal grade, Pierce; store up to several weeks at room temperature over Bio-Rad AG 501-X8 mixed-bed resin)
6 M guanidine·HCl (sequenal grade, Pierce)
HPLC system and 4.6-mm reversed-phase column (see Basic Protocol)

Solubilizing the Protease from Manufacturer Stocks

For sequencing-grade proteases

1a. Dissolve protease (powder) in the shipping vial by adding distilled water to yield a final enzyme concentration of 1 µg/µl.

2a. Aliquot 5- or 10-µl volumes of 1 µg/µl stock solution into 0.5-ml microcentrifuge tubes. Keep one aliquot on ice for testing activity under nonstringent or stringent conditions.

3a. Place remaining aliquots inside a labeled 50-ml tube and store up to 1 year at –20°C.

For proteases purchased in amounts ≥1 mg

1b. Transfer protease to a 1.5-ml microcentrifuge tube. Dissolve powder in water to obtain a final concentration of 5 mg/ml.

2b. Aliquot 200-µl volumes of 5 mg/ml stock solution to 0.5-ml microcentrifuge tubes. Keep one aliquot on ice for further dilution. Place remaining tubes inside a labeled 50-ml tube and store up to 1 year at –20°C.

3b. Dilute remaining 5 mg/ml stock solution with water to a final concentration of 1 mg/ml. Aliquot 5- and 10-µl volumes. Keep one aliquot on ice for testing activity under nonstringent or stringent conditions. Store remaining aliquots as described in step 2b.

IMPORTANT NOTE: *If proteases do not readily dissolve, tap microcentrifuge tube gently and warm <5 min at 37°C. If enzyme still does not dissolve, add TFA to final concentration of 0.1% (v/v). Tap tube gently and warm <5 min at 37°C. Frozen stocks are stable for < 1 year and trypsin may be stable for several years. Do not use 5 mg/ml stocks until 1 mg/ml stocks are depleted. Another tube of concentrated stock can then be thawed, diluted, and aliquoted as above.*

4. Test protease activity under nonstringent conditions (steps 5 through 8) or stringent conditions (step 9 through 15).

It is best to test under stringent conditions. If the enzyme passes that test, it is fine for everything. If it fails, it might still be useful for experiments under nonstringent conditions.

Testing Protease Activity

Test under nonstringent conditions

5. In a 0.5-ml microcentrifuge tube, mix (25 µl total):

5 µl 5 µg/µl cytochrome *c*
16.5 µl water
2.5 µl 10× digestion buffer
1 µl 1 µg/µl enzyme stock.

Vortex tube lightly to mix. Microcentrifuge 10 sec at high speed.

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6. Incubate sample at 37°C for the amount of time recommended for the selected protease (see Table 11.1.1).
7. Analyze ¼ of the sample (500 pmol) by RP-HPLC using a 4.6-mm column (see Basic Protocol step 8).

β-lactoglobulin should be used as substrate for endoproteinase Glu-C, as this protease cannot degrade undenatured cytochrome c. Stocks should be tested periodically (every 6 months) or before a crucial experiment is to take place.

Test under stringent conditions

8. Prepare digests as follows: For trypsin, prepare digestion buffer containing 4 M urea (final); for endoproteinase Lys-C, subtilisin, or thermolysin, prepare a digest mixture containing 7.3 M urea (final) and cytochrome *c* as substrate *or* 2 M guanidine·HCl and triosephosphate isomerase as substrate.
9. Pipet 5 µl of 5 mg/ml cytochrome *c* (2 nmol) or 5 µl of 5 mg/ml triosephosphate isomerase (900 pmol) into a 0.5-ml microcentrifuge tube. Dry in a Speedvac evaporator.
10. Dissolve pellet in 20 µl of 8 M urea or 10 µl of 6 M guanidine·HCl, as follows:

    For 7.3 M urea (final): Add 2 µl 10× buffer
    For 4 M urea (final): Add 15 µl water and 4 µl 10× buffer
    For 2 M guanidine·HCl (final): Add 16 µl water and 3 µl 10× buffer.
11. Allow substrate to denature (see Alternate Protocol 2, steps 1 to 3).
12. Add 1 µl of 1 µg/µl enzyme stock. Vortex sample lightly to mix. Microcentrifuge 10 sec at high speed.
13. Incubate at 37°C for the amount of time recommended for the selected protease (see Table 11.1.1).
14. Analyze ¼ (500 pmol) of the cytochrome *c* digests or ½ (450 pmol) of the triosephosphate isomerase digests by RP-HPLC using a 4.6-mm column (see Basic Protocol, step 8).

Thawing Protease Stock Aliquots for Experimental Use

15. Remove tube containing 1 µg/µl stock solution of desired enzyme from –20°C freezer and place on ice. Allow entire sample to thaw. Tap tube lightly to mix solution and microcentrifuge 10 sec at high speed.
16. Pipet desired volume of stock solution into microcentrifuge tube containing substrate in buffer.
17. If ≤5 µl enzyme remains in stock tube, discard. If >5 µl enzyme remains, recap stock tube, mark cap with black dot, and return to –20°C.

    This tube can be used one more time; always discard any stock protease remaining after second time thawed.

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SUPPORT PROTOCOL 2

REDUCTION AND S-ALKYLATION OF PEPTIDES IN DIGEST MIXTURES

Unless they are reduced, disulfide bonds remain intact during the digestion procedures outlined in the Basic and Alternate Protocols. Several peptides will therefore be disulfide-linked and elute as single peaks during HPLC. In addition, underivatized cysteine residues cannot be identified easily by standard chemical sequencing. Derivatization will also prevent random disulfide bond formation when the reducing agent is removed and peptides are exposed to oxidative conditions.

In this protocol, the peptide mixture is reduced, then S-alkylated with 4-vinylpyridine. The resulting cysteine derivative, pyridylethyl cysteine, is easily detectable during chemical sequencing.

Materials

Peptide digest mixture (see Basic Protocol or Alternate Protocols 1 or 2)
2-mercaptoethanol (2-ME; Bio-Rad): undiluted and 10% (v/v) solution (prepare in Milli-Q water or equivalent immediately before use)
4-vinylpyridine (Aldrich; store at –20°C), undiluted and 10% (v/v) solution (prepare in high-purity grade ethanol immediately before use)
Argon (prepurified)
HPLC system and reversed-phase column (see Basic Protocol)

Reduce sample

1. Add 2 µl of 2-ME per 1 nmol original substrate protein. For smaller quantities of protein (e.g., 100 pmol), add 2 µl of 10% 2-ME.
2. Attach a Pasteur pipet to a length of Tygon tubing connected to the regulator on the argon tank. Adjust argon flow until it is gentle enough to be felt only barely when directed to the back of the hand. Direct argon stream over sample for 20 sec.
3. Cap tube, vortex lightly, and microcentrifuge briefly.
4. Incubate 30 min in 37°C water bath. If S-alkylation is unnecessary (i.e, if the protein will not be sequenced), proceed to step 8.

Alkylate sample

5. Add 6 µl of 4-vinyl pyridine per 1 nmol reduced substrate protein. For smaller quantities of protein (e.g., 100 pmol), add 6 µl of 10% 4-vinylpyridine.

   IMPORTANT NOTE: *Not all lots of 4-vinylpyridine are good, and there is also an aging effect. Quality control should be done on all new batches, and periodically thereafter, by derivatizing a model substrate (e.g., insulin) and checking for pyridylethyl incorporation (by HPLC analysis, monitoring absorbance at 253 nm). Aliquots (20 µl) are then stored under argon at –20°C for one-time use.*

6. Direct stream of argon over sample for 20 sec. Cap tube, vortex lightly, and microcentrifuge briefly.
7. Incubate 30 min at room temperature.

   Do not start alkylation until the HPLC apparatus is completely ready for use (performance-tested and equilibrated at initial conditions); if necessary, the digest should be kept on ice or stored at –70°C prior to alkylation, until HPLC is ready.

Separate peptides by RP-HPLC

8. Inject sample immediately onto RP-HPLC column (see Basic Protocol, step 8).

   HPLC analysis must be performed immediately on S-alkylated samples. Do not let the reaction mixture sit for more than 15 to 20 min after the 30-min incubation, and do not try to store sample at –70°C; either action will severely affect the HPLC profile.

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COMMENTARY

Background Information

Detailed analysis of protein covalent structure usually requires analysis of peptides derived from the larger protein. In contrast to acid-promoted total hydrolysis (e.g., 6 N HCl), peptides are typically generated by selective proteolysis. Endoproteases fragment proteins by cleaving certain peptide bonds, with varying degrees of specificity (Beynon and Bond, 1989; Keil, 1991). In nature, most proteases digest nutrients, aid in cellular defense, or influence other physiological processes. When purified, the proteases with the greatest degree of specificity are useful tools for protein chemical analysis.

Specificities of the most frequently used endoproteases are well established (Keil, 1981, 1991; see Table 11.1.3). When digesting substrates of known sequence, proteases can therefore be rationally selected. The number of fragments resulting from protease digestion will be proportional to the abundance of the targeted peptide bonds in the substrate protein. Average fragment size, on the other hand, is inversely related to the frequency of the targeted peptide bond.

In cases where the protease specificity is determined solely by the P1 or P′1 residue (Table 11.1.3), the number of cleavages depends on the distribution of these particular amino acids. It is estimated that lysine (Lys), arginine (Arg), glutamic acid (Glu), and aspartic acid (Asp) each account for about 5% of all amino acids in the protein databases (Henzel et al., 1994). In contrast, tryptophan (Trp) is less abundant and glycine (Gly) more abundant. Thus, a tryptic or an endoproteinase Lys-C digest should yield about 11 or 6 fragments per 100 residues, respectively.

Enzymatic digestion of a protein results in a pool of variously sized peptides. The complexity of the mixture is correlated to the molecular weight of the substrate. Although some information may be obtained by analyzing the mixture directly (e.g., by mass spectrometry), it is standard procedure to first perform some type of fractionation (e.g., reversed-phase (RP)-HPLC).

The combined technique of digestion followed by separation is commonly called "peptide mapping," particularly when used for comparative purposes. Because RP-HPLC can detect differences as small as a few carbon or oxygen atoms, peptides that differ by a single amino acid or by a post-translational modification (either naturally occurring or experimentally induced) will elute with different retention times (i.e., at a different position in the peptide map). In principle, single amino acid substitutions or modifications can be detected and the distinct peptides isolated. More detailed information about molecular composition is then obtained from peptide sequencing and mass analysis. If the sequence of the substrate protein is known, any residue of interest can be precisely located.

Similarly, sites of phosphorylation (Chapter 13), glycosylation (Chapter 12), or other post-translational modifications can be located as long as the unmodified protein is digested as a control. Modification can be induced in vivo or performed in vitro. If a protein is known to be constitutively modified in vivo, the modification can be selectively removed from the "control" protein (e.g., by phosphatases or endoglycosidases). Peptide mapping is facilitated when the modifying group is also detectable by radioisotopic or spectrophotometric monitoring.

Peptide mapping can also be used to detect and localize a protein disulfide array. In this case, reduced and unreduced peptides will be differently positioned in the comparative map, enabling identification of paired cysteines. Finally, mapping is also a preferred tool to verify the authenticity of overexpressed proteins (Christianson and Paech, 1994).

When digesting proteins of unknown sequence, fragmentation was traditionally performed, not for mapping purposes, but to generate sets of overlapping peptide sequences (e.g., tryptic and chymotryptic peptide digests) essential for deducing the full protein structure (Tempst and Van Beeumen, 1983). With the advent of DNA cloning technology, internal peptide sequences facilitate conventional and PCR-based cloning of the corresponding genes, which can then be easily sequenced (Tempst et al., 1990). Moreover, "internal" sequencing is most often required to overcome the problem of blocked N-termini, a major obstacle for protein chemical analysis.

Efficient proteolytic cleavage is often hampered or prevented due to the insolubility or protease resistance of the substrates. A rather large percentage of membrane proteins and secreted proteins present these problems. Denaturing or chemically modifying the substrate protein can improve digestion efficiency by enhancing solubility or breaking up any tightly packed structures. However, such manipula-

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Table 11.1.3 Specificity of Selected Proteolytic Enzymes[a]

Enzyme	Specificity	Comments
Trypsin	P_1 = Arg > Lys	Inhibited with P_2 ----- P_1 ------ ↓ ---- P_1' (1) X ----- Arg/Lys -- ↓ ---- Pro (2) Asp ---- Lys ----- ↓ ---- Asp (3) Arg ---- Arg ----- ↓ ---- Arg/His Reduced cleavage seen with (1) X ----- Lys ----- ↓ ---- Asp/Glu (2) X ----- Arg ----- ↓ ---- Asp (3) Asp ---- Lys ----- ↓ ---- Asn/Gln/His/Phe/Leu (4) Asp ---- Arg ----- ↓ ---- Glu/His (5) X ----- Arg/Lys -- ↓ ---- Arg/Lys (6) Arg ---- Arg/Lys -- ↓ ---- X (7) Lys ---- Lys ----- ↓ ---- X (8) Lys ---- Arg ---- ↓ ---- His
Endoproteinase Lys-C (KC)	P_1 = Lys	Not inhibited when P_1' = Pro
Endoproteinase Glu-C	P_1 = Glu >>> Asp	Reduced cleavage with P_1' = Leu/Phe or with P_1 surrounded by Glu/Asp Ammonium bicarbonate strongly inhibits, making it selective for the Glu-X bond under standard digest conditions. Adding more enzyme or extending incubation times might help cleave at Asp. In phosphate buffer, Asp-X bonds cleaved (at a 15% rate of Glu-X bonds).
Endoproteinase Asp-N	P_1' = Asp	—
Chymotrypsin	P_1 = Trp > Tyr > Phe >> Leu > Met >> His >>> Asn/Gly	Cleavage after Arg/Lys possible, but may be unspecific or the result of contaminating trypsin Inhibited with P_1' = Pro Enhanced cleavage seen with (1) P_3, P_1', P_2', or P_3' = Arg (Lys) (2) P_2 = Pro Reduced cleavage seen with (1) P_2, P_1', or P_2' = Asp (Glu) (2) P_3 or P_2' = Pro
Subtilisin	P_1 = neutral or acidic amino acid (broad specificity)	Enhanced cleavage seen when (1) P_1' = Gly (2) P_2 = bulky/hydrophobic amino acid
Thermolysin	P_1' = Leu/Ile > Phe > Val >> Tyr > Ala	Inhibited with P_2' = Pro; not inhibited with P_1 = Pro Enhanced fragmentation with P_1 = Phe/Tyr/Trp Reduced fragmentation with P_1 = Glu/Asp
Pepsin	P_1 or P_1' = Phe >> Leu >> Trp > Ala > other hydrophobic amino acids	—
Elastase	P_1 or P_1' = Ile > Val > Ala Gly/Ser (and other neutral, nonaromatic amino acids)	—
Papain	P_2 = hydrophobic amino acid	Very broad specificity; extensive degradation Enhanced fragmentation with P_1 = Lys/Arg

[a]Cleavage site nomenclature: P_3 - - - P_2 - - - P_1 - - - ↓ - - - P_1' - - - P_2' - - - P_3', where ↓ marks the site of cleavage.

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tions (e.g., reduction and alkylation in the presence of guanidine·HCl, followed by dialysis or chromatography) are tedious and frequently result in heavy losses, particularly when working with protein quantities <1 nmol. Moreover, some unstable chemical groups (e.g., carbetoxy histidines) or bonds (e.g., disulfides) get destroyed in the process and can therefore no longer be mapped.

Instead, it is easier to supplement the digestion buffer with agents that promote substrate solubility and denature the protein without interfering with protease activity. For this reason, protocols for protease digestion in the presence of organic solvents, detergents, urea, and guanidine·HCl have been developed (see Alternate Protocol 1 and Alternate Protocol 2). Further advantages of such approaches include improved ability to dissolve protein pellets, avoidance of the need to remove additives present after chromatography or electrophoresis, increased reproducibility (e.g., in chaotrope solutions), and limited proteolysis of solubilized proteins in their native state (e.g., in nondenaturing detergents such as CHAPS).

Critical Parameters and Troubleshooting

In practice, with many substrates, proteolytic digests under nondenaturing conditions are essentially a waste of time; they usually don't work (see Table 11.1.2). Unless special reasons exist to subject a protein to proteolytic attack in its native state, it is advisable to include urea or guanidine·HCl in the digest mixture by default. Provided that the conditions summarized in Table 11.1.1 are followed, and enzymes and buffer components are of good quality (and have been stored correctly), the failure rate for protease digestion will be minimal. Access to a well-maintained HPLC system is also prerequisite.

Enzymes and buffer components

Enzyme activity must be assessed when a new lot (or brand) is received, and then periodically thereafter, either after prolonged storage or whenever concentrated stock solutions are diluted and aliquoted (see Support Protocol 2). The suppliers and grades that have worked consistently well in our hands are listed in the Materials section of Support Protocol 1. Repeated cycles of freezing and thawing should be avoided by preparing small (single-use) aliquots (see Support Protocol 1). Activities should be checked under conditions at least as stringent as those of the planned experiment, preferentially using protease-resistant substrates (e.g., superoxide dismutase or triosephosphate isomerase). All endoproteases should digest cytochrome *c* quite easily under nondenaturing conditions, except endoproteinase Glu-C which works best on β-lactoglobulin (see Table 11.1.2). If any of these low-stringency quality control tests fail, the investigator should complain to the enzyme's manufacturer and demand a new batch at no extra charge.

All buffer components must be "ultra-pure" or "reagent-grade" quality, and the water of Milli-Q purity. Guanidine·HCl solutions must always be made fresh; many problems have been associated with solutions that have aged for only a few days. Urea solutions can be stored up to several weeks at room temperature over Bio-Rad AG 501-X8 mixed-bed resin to capture any cyanates that might form with time. Do not use the blue indicator dye ("D") form of the resin, as the dye has been found to leach out into the urea and interfere with RP-HPLC. Newly prepared stock solutions should be tested with enzyme batches previously found to work well.

Protease compatibilities

Endoproteases have been systematically tested in the presence of increasing concentrations of urea, guanidine·HCl, SDS, nonionic and zwitterionic detergents, and acetonitrile and isopropanol, using cytochrome *c* and β-lactoglobulin as model substrates (Riviere et al., 1991; L.R.R. and P.T., unpub. observ.). The highest concentrations of additives that still permit a fairly complete digest are listed in Table 11.1.1. Cytochrome *c* peptide maps resulting from these digests are shown in Fig. 11.1.1. It should be noted that the conditions listed for digests containing urea and guanidine·HCl have been carefully optimized. Small changes may result in a drastically different outcome of an experiment, and, if so desired, modified conditions should be tested prior to the real experiment. It is advisable to not change conditions within any set of comparative peptide mapping experiments.

In the presence of 8 M urea, Tris·Cl buffers work best for endoproteinase Lys-C and subtilisin digestion, tending to give higher yields and fewer partial cleavages than bicarbonate buffers. Both types of buffer are equally compatible with thermolysin digestion in 8 M urea, although Tris·Cl digests consistently generate larger fragments. Trypsin activity in 4 M urea is borderline and requires the presence of Ca^{2+} and a lengthy (24-hr) incubation to ensure even

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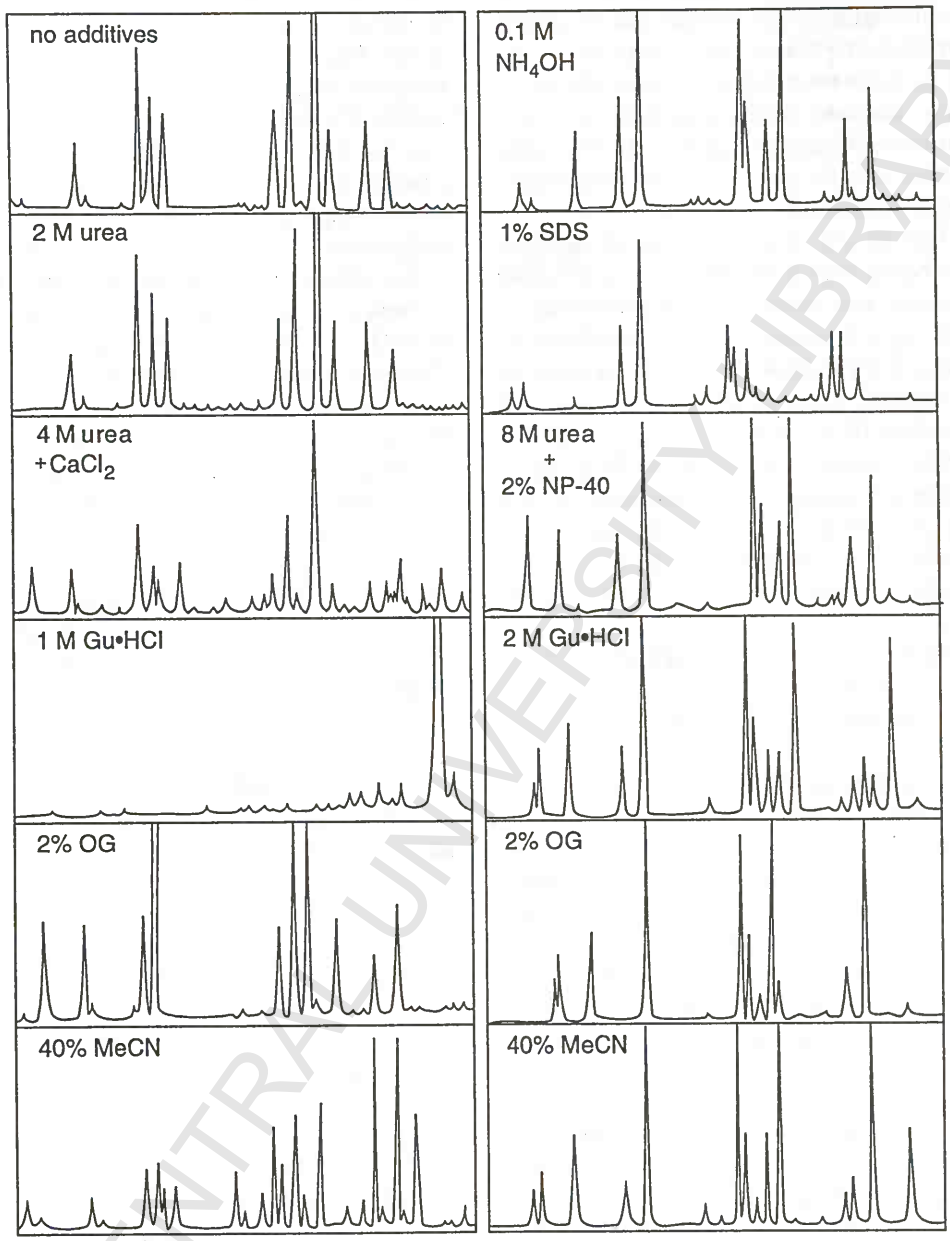

Figure 11.1.1 Effects of additives on proteolytic digestion of 1 nmol of cytochrome *c* with 1 µg trypsin (left) or 1 µg endoproteinase Lys-C (right). Digest conditions are listed on the panels. Digest mixtures were separated by reversed-phase HPLC on a 4.6-mm Vydac C18 column, using gradient conditions and flow rate given in the Basic Protocol. Abbreviations: MeCN, acetonitrile; OG, octyl glucoside.

partial digestion (Fig. 11.1.1). In general, artificial blocking (i.e., carbamylation) of the newly generated N-termini during digestion in the presence of urea is not evident, as determined by sequencing.

Whereas endoproteinase Lys-C and subtilisin work well in the presence of 2 M guanidine ·HCl, chymotrypsin does so only at higher bicarbonate concentrations and after prolonged incubation (24 hr). Trypsin does not digest proteins in guanidine·HCl solutions because guanidine is a competitive inhibitor of the enzyme (Fig. 11.1.1). Some batches of trypsin, less pure than the Boehringer-Mannheim or Promega sequencing grades, have been found to work under these conditions. However, lim-

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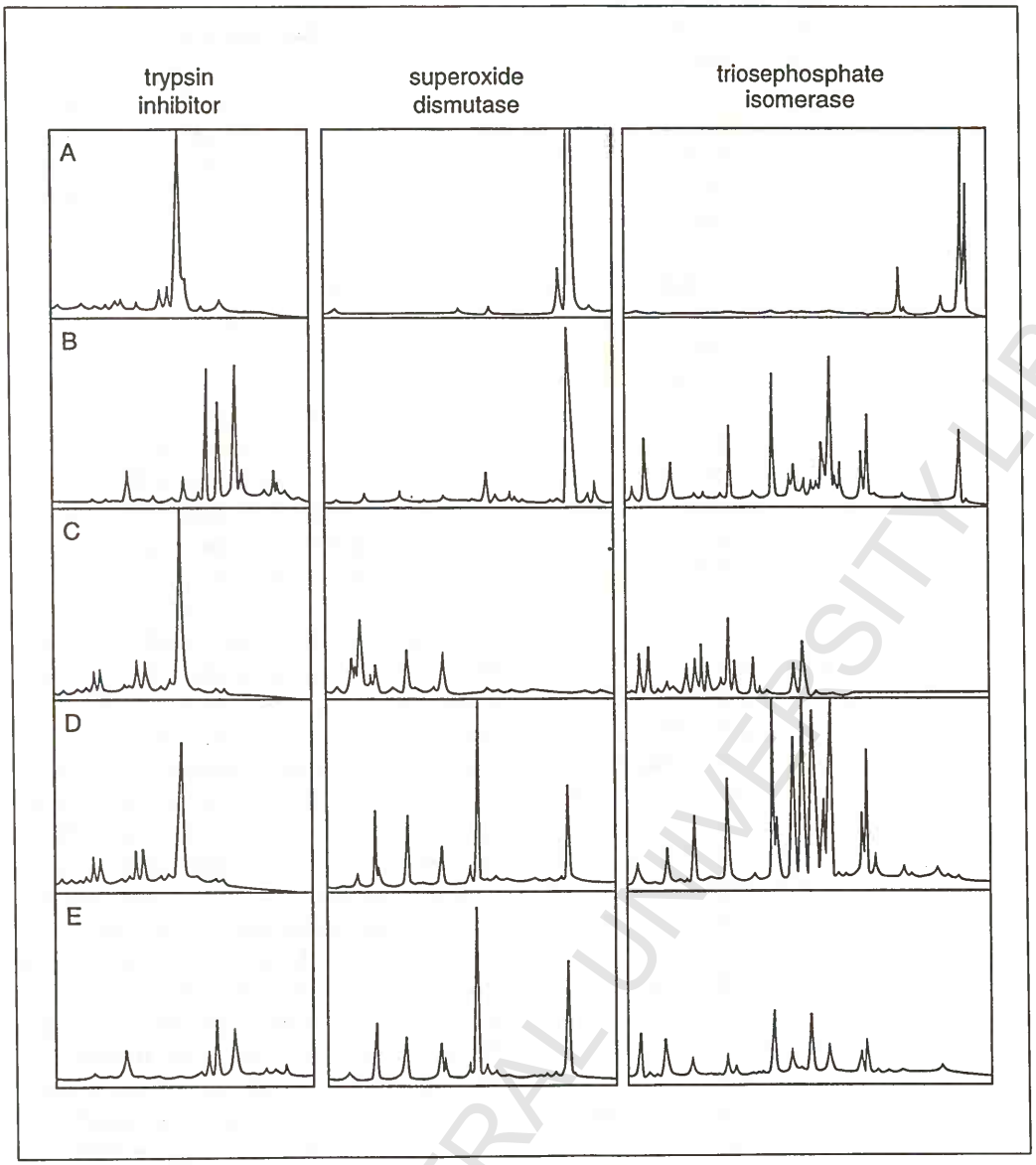

Figure 11.1.2 Enzymatic digestion of protease-resistant substrates. 2 nmol trypsin inhibitor (left panels), 1 nmol superoxide dismutase (middle panels), and 1 nmol triosephosphate isomerase (right panels) were digested by 1 µg endoproteinase Lys-C. Digestion conditions were as follows: (**A**) no additives; (**B**) 8 M urea; (**C**) 1 M guanidine·HCl (**D**) 2 M guanidine·HCl; (**E**) 1% SDS (with guanidine·DS precipitation). Digest mixtures were separated by reversed-phase HPLC on a 4.6-mm Vydac C18 column, using gradient conditions and flow rate as given in the Basic Protocol.

ited sequencing of the resulting peptides indicated chymotryptic-like cleavages.

Although enzymatic digestion in the presence of SDS has been described (Cleveland et al., 1977), it has not found widespread use in combination with HPLC, primarily because dodecyl sulfate interferes with peptide separation on reversed-phase packings. Excess SDS can be removed by guanidine·HCl precipitation (as guanidine·DS salt) prior to RP-HPLC. Chymotrypsin and endoproteinase Glu-C retain activity in 0.1% SDS. Although endo Glu-C digests are usually incomplete, chymotryptic digestion is quantitative but restricted (i.e., only larger fragments are reproducibly generated). If the substrate is first heated at 95°C, endoproteinase Lys-C and subtilisin can degrade almost any protein in 1% SDS (Fig. 11.1.2). Recovery after guanidine·DS precipitation is highly variable, with the worst yields for larger or hydrophobic peptides (Figs. 11.1.1 and 11.1.2). This is not a problem with SDS concentrations ≤0.1%.

With few exceptions, enzymatic proteolysis works quite well in 2% nonionic or zwitterionic detergents, and in acetonitrile or isopropanol

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at concentrations up to 40%. Endoproteinase Lys-C even works in 8 M urea/2% NP-40, a very strong solubility-promoting combination. When heated, several proteins precipitate in organic solvent concentrations over 40%. Very detailed studies on digestion with organic solvents have been described by Welinder (1988).

Under certain conditions, digests are restricted; i.e., there are fewer peaks than usual but all in good yields. Combinations of enzymes and additives that yield restricted digests are indicated in Table 11.1.1. With no exceptions, even with enzymes of low specificity (e.g., subtilisin, thermolysin, and pepsin), peptide patterns are very reproducible under a strictly controlled set of conditions. All enzymes and additives can therefore be used in peptide mapping experiments.

Additional experimental parameters

Enzymatic proteolysis is generally done at 37°C. However, most enzymes work fairly well in the 20° to 50°C range. Optimal temperature for thermolysin activity is actually 60°C, but this offers no apparent advantage over its use at 37°C. Whereas pH optima of most proteases are quite narrow, considerable flexibility exists for subtilisin and endoproteinase Lys-C digests, which can proceed at pH 10.5 (Table 11.1.1). Reaction times listed in Table 11.1.1 are usually adequate. Prolonged incubation provides absolutely no advantage. If no favorable result is obtained at the end of the indicated time periods, something is wrong with the experiment or the chosen combination of enzyme and denaturant is not optimal for the particular substrate. The former situation can be monitored by performing the proper control digests. The latter can be alleviated by trying a different combination of enzyme and denaturant.

To ensure complete digestion, the final enzyme concentrations should be >1 μg/100 μl of reaction mixture under native conditions and >1 μg/50 μl of reaction mixture in the presence of guanidine·HCl, urea, or SDS. This restriction essentially determines the enzyme/substrate ratios, with the clear rule that those ratios should be kept as low as possible to avoid excessive autolytic digestion of the protease. Finally, it is imperative that the substrate be in solution before the protease is added. Never try to improve solubility (e.g., by heating, sonicating, or vortexing) in the presence of the enzyme, as this will result in loss of activity.

Protease-resistant substrates

Proteases active in 2 M guanidine·HCl and 8 M urea provide efficient tools to digest substrates whose physical properties prevent normal enzymatic degradation. A brief survey of the literature along with additional tests indicate that ribonuclease, ADP-ribosyl cyclase, lysozyme, amylase, superoxide dismutase, triosephosphate isomerase, xylose isomerase, pancreatic trypsin inhibitor, and many other proteins are quite resistant to protease digestion (see Table 11.1.2). After heating in 6 M guanidine·HCl and subsequent dilution to 2 M urea, they apparently do not refold to a compact structure, rendering them amenable to digestion. Alternatively, 8 M urea can be used to denature the protein. All the protease-resistant substrates listed above have been digested successfully using one or the other technique, but not always by both (Vangrysperre et al., 1989; Fig. 11.1.2). When the guanidine·HCl or urea concentrations are lowered to 1 M or 4 M, respectively, the digests no longer proceed. This excludes the use of endoproteinase Glu-C and trypsin for these purposes. In general, guanidine·HCl in combination with endoproteinase Lys-C is the method of choice.

Endoproteinase Lys-C and subtilisin undergo a substantial amount of autolysis in 2 M guanidine·HCl, 8 M urea, or 1% SDS. Care must be taken to not confuse these peaks with the real peptide map. Autolysis profiles are fairly reproducible. Thus, with enzyme/substrate ratios of ≤1:10 and appropriate enzyme blank experiments, mistakes can usually be avoided. Should an autolytic fragment be accidentally analyzed by sequencing or mass spectrometry, the error can be quickly traced by comparing the sequence with the known sequence of the protease (Table 11.1.4).

Limited and partial digestion

Although the usual goal of a proteolytic digest is to fully cleave all susceptible bonds and generate a complete peptide map, sometimes restricted digestion is advantageous. Cleavage of an artificially low number of bonds, each one to completion, will yield fewer (and bigger) fragments and result in less complicated chromatograms. Addition of chaotropes will sometimes lead to exactly such an effect. Mild digestion of a protein in its native state may provide useful information on the domain structure and surface topography (Marks et al., 1990). The protein is thereby kept soluble in a nondenaturing detergent (e.g., CHAPS) solu-

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Table 11.1.4 Protease Sequences

Enzyme	Sequence
α-Trypsin (bovine)	
Chain 1	IVGGYTCGAN TVPYQVSLNS GYHFCGGSLI NSQWVVSAAH CYKSGIQVRL
	GEDNINVVEG NEQFISASKS IVHPSYNSNT LNNDIMLIKL KSAASLNSRV
	ASISLPTSCA SAGTQCLISG WGNTK (125)
Chain 2	SSGTSYPDVL KCLKAPILSD SSCKSAYPGQ ITSNMFCAGY LEGGKDSCQG
	DSGGPVVCSG KLQGIVSWGS GCAQKNKPGV YTKVCNYVSW IKQTIASN (98)
α-Trypsin (pig)	
Chain 1	IVGGYTCAAN SIPYQVSLNS GSHFCGGSLI NSQWVVSAAH CYKSRIQVRL
	GEHNIDVLEG NEQFINAAKI ITHPNFNGNT LDNDIMLIKL SSPATLNSRV
	ATVSLPRSCA AAGTECLISG WGNTK (125)
Chain 2	SSGSSYPSLL QCLKAPVLSD SSCKSSYPGQ ITGNMICVGF LEGGKDSCQG
	DSGGPVVCNG QLQGIVSWGY GCAQKNKPGV YTKVCNYVNW IQQTIAAN (98)
Endoproteinase Lys-C (*Achromobacter lyticus* strain M497-1)	GVSGSCNIDV VCPEGDGRRD IIRAVGAYSK SGTLACTGSL VNNTANDRKM
	YFLTAHHCGM GTASTAASIV VYWNYQNSTC RAPNTPASGA NGDGSMSQTQ
	SGSTVKATYA TSDFTLLELN NAANPAFNLF WAGWDRRDQN YPGAIAIHHP
	NVAEKRISNS TSPTSFVAWG GGAGTTHLNV QWQPSGGVTE PGSSGSPIYS
	PEKRVLGQLH GGPSSCSATG TNRSDQYGRV FTSWTGGGAA ASRLSDWLDP
	ASTGAQFIDG LDSGGGTP (268)
Endoproteinase Glu-C (*Staphylococcus aureus* strain V8)	VILPNNDRHQ ITDTTNGHYA PVTYIQVEAP TGTFIASGVV VGKDTLLTNK
	HVVDATHGDP HALKAFPSAI NQDNYPNGGF TAEQITKYSG EGDLAIVKFS
	PNEQNKHIGE VVKPATMSNN AETQVNQNIT VTGYPGDKPV ATMWESKGKI
	TYLKGEAMQY DLSTTGGNSG SPVFNEKNEV IGIHWGGVPN EFNGAVFINE
	NVRNFLKQNI EDIHFANDDQ PNNPDNPDNP NNPDNPNNPD EPNNPDNPNN
	PDNPDNGDNN NSDNPDAA (268)
Chymotrypsin A (bovine)	
A chain	CGVPAIQPVL SGL (13)
B chain	IVNGEEAVPG SWPWQVSLQD KTGFHFCGGS LINENWVVTA AHCGVTTSDV
	VVAGEFDQGS SSEKIQKLKI AKVFKNSKYN SLTINNDITL LKLSTAASFS
	QTVSAVCLPS ASDDFAAGTT CVTTGWGLTR (130)
C chain	YTNANTPDRL QQASLPLLSN TNCKKYWGTK IKDAMICAGA SGVSSCMGDS
	GGPLVCKKNG AWTLVGIVSW GSSTCSTSTP GVYARVTALV NWVQQTLAAN (100)

tion and the enzyme activity attenuated through reduced enzyme/substrate ratios (e.g., <1:100), lower digestion temperatures (≤20°C), and shorter incubation times (≤30 min). Fragments can be separated by HPLC or SDS-PAGE.

In contrast to experimentally designed limited proteolysis, partial digestions also occur quite often unintentionally. The problem derives from incomplete (10% to 90%) cleavage of certain peptide bonds which, through combinatorial overlaps, results in many more peptides than expected and lower yields. Partial digestions are typically caused by incompletely denatured substrates or proteases with low activity. The necessary corrective measures are self-evident.

Preventing failures

Provided that the fractionation techniques were carried out properly, the absence of a peptide profile during HPLC or after SDS-PAGE indicates that the digest didn't work or that there was a lack of sufficient substrate to begin with. The protein could have been lost

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during final preparation (e.g., dialysis or precipitation) or transfer, or not been properly solubilized. The quantity of the starting substrate should be estimated by SDS-PAGE. Other factors that may contribute to failure include inactive proteases, the presence of protease inhibitors (carried over from the protein purification procedure), an incorrect pH, or a concentration of denaturants that was either too low (so that the substrate refolded) or too high (so that the protease was inactive). Preliminary testing of enzyme and digest conditions on model substrates, and pilot optimization experiments using dispensable amounts of the "real" protein, will largely prevent such mishaps from occurring. In addition, when a preparative experiment is carried out, analyzing 5% to 10% of the sample, while keeping the rest on ice or frozen, prior to preparative chromatography or electrophoresis will prevent a preparative "total loss" in case something goes wrong unexpectedly. It should be noted that undigested guanidine·HCl–denatured protein cannot usually be recovered from reversed-phase columns, because the exposed hydrophobic core of the protein becomes irreversibly adsorbed. This could lead the investigator to conclude that there was no protein present at the onset of the experiment.

Anticipated Results

In the basic protocol, 100 pmol of cytochrome *c* digested with any of the enzymes listed in Table 11.1.1 will yield a nice peak profile after separation on a 4.6-mm C4 or C18 reversed-phase column, with an absorbance detector setting of 0.05 AUFS at 214 nm. Fifty pmol may be digested and separated on a 2.1-mm column. Digestion in the presence of chaotropes and SDS requires about twice the amount of starting material.

When substrate protein is available in quantities <100 pmol, in situ digests are more efficient than those in solution, both in terms of sample preparation and experimental losses. The drawback is that not all peptides are recovered, which makes in situ procedures less than ideal for peptide mapping experiments.

Time Considerations

Time required for enzymatic digestion of protein sample in solution can be broken down as follows: dissolving and denaturing the sample should take 15 to 45 min; preparing solutions for the digest will take 20 min; protease digestion requires from 2 to 24 hr; reduction and alkylation of sample will take 1 hr; and HPLC separation takes 1 to 1.5 hr. The entire procedure can be completed in 1 day (for the shorter digest times), but enzymes, reagents, and the HPLC apparatus should be checked the day before. The HPLC should be checked again using a standard peptide mixture a few hours before running the actual digest mixture.

A set of samples may be digested simultaneously, with the restriction that only three or four HPLC experiments can be completed in one (long) day on one instrument using manual collection. This is a critical consideration when peptide mixtures are S-alkylated with 4-vinylpyridine (because they cannot be stored). Underivatized analytical digests can be processed using an autosampler.

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Enzymatic Digestion of Proteins on PVDF Membranes

Enzymatic digestion of membrane-bound proteins is one of the most widely used procedures for determining the internal amino acid sequence of proteins that either have a blocked amino terminus or require two or more stretches of sequence data for DNA cloning or confirmation of protein identification. Because the final step of protein purification is usually SDS-PAGE, electroblotting to either polyvinylidene difluoride (PVDF) or nitrocellulose is the simplest and most common procedure for recovering protein free of contaminants (e.g., SDS or acrylamide) with a high yield. As described in this unit, PVDF is preferred over nitrocellulose because it can be used for a variety of other structural analysis procedures, such as amino-terminal sequence analysis and amino acid analysis. In addition, peptide recovery from PVDF membranes is higher than from nitrocellulose, particularly from higher-retention PVDF (e.g., ProBlott, Transblot, Westran, or Immobilon P^{sq}). Finally, PVDF-bound protein can be stored dry, as opposed to nitrocellulose-bound protein, which must remain wet during handling and storage to prevent loss of peptides during digestion.

DIGESTION OF PVDF-BOUND PROTEINS IN A HYDROGENATED TRITON X-100 BUFFER

BASIC PROTOCOL

This procedure describes enzymatic digestion of proteins bound to PVDF following SDS-PAGE (*UNITS 10.1-10.4*) and electroblotting (*UNIT 10.7*). PVDF-bound proteins are visualized by staining and protein bands are excised from the membrane and placed directly in hydrogenated Triton X-100 (RTX-100) digestion buffer. RTX-100 acts both as an elution reagent for removing peptides from the membrane and as a blocking reagent that prevents any proteinase from adsorbing to the PVDF membrane. After a 24-hr incubation with a proteinase, the peptide fragments are recovered from the digestion buffer. Further washes of the membrane remove any remaining peptides, and the pooled digestion buffer and washes can be analyzed by reversed-phase HPLC (*UNIT 11.6*). The amino acid sequence can then be determined by either gas-phase or pulsed liquid-phase sequence analysis.

NOTE: The key to success with this procedure is cleanliness. Use of clean buffers, tubes, and staining/destaining solutions, as well as only hydrogenated Triton X-100, greatly reduces contaminant peaks during reversed-phase HPLC. A corresponding blank piece of membrane must always be analyzed at the same time a sample is digested, as a negative control.

Materials

Protein samples electrophoresed on SDS-polyacrylamide gel (*UNITS 10.1-10.4*)
PVDF membrane (e.g., ProBlott, Immobilon P^{sq}, or Westran)
Milli-Q water or equivalent
Digestion buffer (see recipe and Table 11.2.1)
Appropriate 0.1 µg/µl enzyme solution (depending on the cleavage site required; see recipe)
0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water or equivalent
1% (v/v) diisopropylfluorophosphate (DFP) in ethanol (see recipe)
Powder-free gloves
Glass plate
Forceps
Sonicating water bath
Injection vials for HPLC

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Table 11.2.1 Digestion Buffers for Various Enzymes[a]

Enzyme	Digestion buffer	Recipe	Comments
Trypsin or endoproteinase Lys-C	1% RTX-100/10% acetonitrile/100 mM Tris·Cl, pH 8.0	100 µl 10% RTX-100 stock 100 µl acetonitrile 300 µl HPLC-grade water 500 µl 200 mM Tris·Cl, pH 8.0	RTX-100 prevents enzyme adsorption to membrane and increases recovery of peptides
Endoproteinase Glu-C	1% RTX-100/100 mM Tris·Cl, pH 8.0	100 µl 10% RTX-100 stock 400 µl HPLC-grade water 500 µl 200 mM Tris·Cl, pH 8.0	Acetonitrile is omitted because it decreases digestion efficiency of endoproteinase Glu-C
Clostripain	1% RTX-100/10% acetonitrile/2 mM DTT/1 mM $CaCl_2$/100 mM Tris·Cl, pH 8.0	100 µl 10% RTX-100 stock 100 µl acetonitrile 45 µl 45 mM DTT 10 µl 100 mM $CaCl_2$ 245 µl HPLC-grade water 500 µl 200 mM Tris·Cl, pH 8.0	DTT and $CaCl_2$ are necessary for clostripain activity

[a]See *APPENDIX 2E* for Tris·Cl and DTT stock solution recipes. Abbreviations: DTT, dithiothreitol; RTX-100, hydrogenated Triton X-100 (Tiller et al., 1984).

Microbore HPLC

Clean capless 1.5-ml microcentrifuge tubes and separate caps (e.g., Sarstedt)

Additional reagents and equipment for electroblotting (*UNIT 10.7*), staining transferred proteins (*UNIT 10.8*), and reversed-phase HPLC (*UNIT 11.6*)

Transfer sample to PVDF membrane and stain protein bands

1. Electroblot proteins from polyacrylamide gel to PVDF membrane. For most efficient transfer, follow protocol for tank-transfer system rather than semi-dry system.

   When separating the protein sample by SDS-PAGE, concentrate as much protein into each lane as possible; however, if protein resolution is a concern, up to 20 individual bands of protein (~ 5cm^2) can be excised and combined in a single proteinase digestion reaction.

2. Stain PVDF membrane with Ponceau S, amido black, India ink, or Coomassie brilliant blue-G. Destain membrane until background is clean enough to visualize stained protein bands. Rinse destained membrane three times with distilled water to remove excess acetic acid.

   Ponceau S and amido black are preferred because they generally contain the fewest contaminants. Most commercial sources of Coomassie brilliant blue contain a large amount of contaminating material and should be avoided. If using Coomassie brilliant blue, we have found that preparations with greater than 90% dye content (e.g., from Aldrich) is suitable.

3. Excise desired protein bands from membrane and place in 1.5-ml microcentrifuge tube. Also excise a blank region of membrane, approximately the same size as protein band, to serve as a negative control.

   The negative control should be cut from the same blot as the protein sample and should have undergone the same manipulations (e.g., electroblotting, staining, destaining).

4. Air dry PVDF-bound protein at room temperature and store at –20° or 4°C.

   IMPORTANT NOTE: *If nitrocellulose must be used, the membrane-bound protein must be stored wet at –20°C and never allowed to completely dry out. If the membrane dries, peptide recovery can be greatly diminished.*

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Digest membrane-bound protein with appropriate proteinase

IMPORTANT NOTE: Gloves should be worn during all steps to avoid contamination of membrane with skin keratins.

5. Place ~100 µl Milli-Q water onto a clean glass plate. Submerge membrane samples into water droplet and transfer wet membrane to a dry area of the plate. Cut membrane lengthwise into 1-mm-wide strips with a clean razor blade. Cut membrane strips into 1 × 1–mm squares. Treat negative control using identical conditions as the samples.

   Keeping the membrane wet will simplify manipulation of the sample as well as minimize any static charge, which could cause PVDF membrane to "jump" off the plate. The 1 × 1–mm pieces of membrane will settle to the bottom of the tube and require less digestion buffer to completely immerse the membrane.

6. Slide membrane squares onto forceps and place in a clean 1.5-ml microcentrifuge tube.

   Use the cleanest tubes possible to minimize contamination during peptide mapping. Surprisingly, many UV-absorbing contaminants can be found in microcentrifuge tubes, and this appears to vary with supplier and lot number. Tubes can be cleaned by rinsing with 1 ml HPLC-grade methanol followed by 2 rinses with 1 ml Milli-Q water prior to adding the protein band.

7. Add 50 µl of appropriate digestion buffer to membrane squares. Vortex sample 10 to 20 sec and incubate 5 to 30 min at room temperature. Add 50 µl digestion buffer to an empty microcentrifuge tube to serve as an RTX-100 blank for HPLC analysis (optional).

   Digestion buffers for typical proteases are listed in Table 11.2.1. The amount of digestion buffer can be increased or decreased depending on the amount of membrane; however, the best results will be obtained with a minimum amount of digestion buffer. PVDF membrane will float in the solution at first, but will submerge after a short time, depending on the type and amount of PVDF.

8. Add a volume of 0.1 µg/µl enzyme solution that yields an enzyme-to-substrate ratio of ~1:10 (w/w). Incubate samples (including RTX-100 blank) 22 to 24 hr at 37°C.

   The amount of substrate protein on membrane can be estimated by comparing staining intensity of band to known quantities of stained standard proteins. The 1:10 ratio of enzyme to substrate is a general guideline. Ratios of 1:2 through 1:50 can be used without loss of enzyme efficiency or peptide recovery. An enzyme that will produce peptides greater than 10 amino acids in length should be selected. Prior amino acid analysis (UNIT 3.2) is helpful for estimating the number of cleavage sites in the protein. A second aliquot of enzyme may be added after 4 to 6 hr.

Extract the digested peptides

9. Vortex membrane sample 5 to 10 sec and sonicate 5 min at full speed in a sonication bath. Microcentrifuge sample 2 min at ~4000 rpm (1800 × *g*), room temperature. Transfer supernatant to an HPLC injection vial.

10. Add another 50 µl of digestion buffer to the membrane in the 1.5-ml centrifuge tube and repeat step 9. Pool supernatant with original buffer supernatant in HPLC vial.

11. Add 100 µl of 0.1% TFA to membrane, vortex, and transfer supernatant to HPLC vial (total volume of samples should be 200 µl). Add 150 µl digestion buffer to RTX-100 blank to bring final volume to 200 µl.

    Most of the peptides (~80%) are recovered in the original digestion buffer; however, these additional washes ensure maximum recovery of peptides from the membrane.

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12. Terminate enzymatic digestion reaction by immediately analyzing sample on HPLC or by adding 2 µl of 1% DFP solution to sample.

CAUTION: *DFP is a dangerous neurotoxin and must be handled with double gloves under a chemical fume hood. Please follow all precautions listed for this chemical.*

Analyze samples by reversed-phase HPLC

13. Inspect pooled supernatants for small pieces of membrane or particles that might clog HPLC tubing. Remove any membrane fragments with a clean probe such as pointed tweezers, wire, or long pipet tip. Alternatively, microcentrifuge sample 2 min at 4000 rpm (1800 × *g*), room temperature. Transfer supernatant to a clean vial.

A precolumn filter will help increase the life of HPLC columns, which frequently have problems with clogged frits.

14. Analyze samples in a microbore HPLC. Collect fractions in capless 1.5-ml microcentrifuge tubes. Cap samples and store at –20°C until ready for sequencing.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Diisopropylfluorophosphate (DFP) solution, 1%

Add 10 µl DFP to 990 µl absolute ethanol in a capped microcentrifuge tube. Store at –20°C (stable at least 1 month).

CAUTION: DFP is a dangerous neurotoxin. Handle with double gloves in a chemical fume hood. Please follow all precautions listed with this chemical.

Digestion buffer

Prepare digestion buffer in 1-ml aliquots according to recipes listed in Table 11.2.1. Store at –20°C up to 1 week.

NOTE: *Use only hydrogenated Triton X-100 (10% RTX-100 solution, protein grade; Calbiochem) because UV-absorbing contaminants present in ordinary Triton X-100 make identification of peptides on subsequent HPLC analysis impossible.*

Enzyme solutions

Trypsin: Solubilize 25 µg trypsin (sequencing grade, Boehringer Mannheim) in 25 µl of 0.01% trifluoroacetic acid (TFA) and let stand ~10 min. Transfer 5-µl (5 µg) aliquots into clean microcentrifuge tubes, dry in a Speedvac evaporator, and store at –20°C (stable at least 1 month). Immediately before use, reconstitute enzyme in 50 µl of 0.01% TFA to obtain a 0.1 µg/µl working solution.

Trypsin cleaves at arginine and lysine residues. One aliquot provides enough enzyme to digest 50 µg of protein.

Endoproteinase Lys-C: Solubilize 3 U endoproteinase Lys-C (Boehringer Mannheim) in 200 µl Milli-Q water to obtain a 0.015 U/µl working solution. Let stand ~10 min. Transfer 5-µl aliquots into clean tubes and store at –20°C (stable at least 1 month).

Endoproteinase Lys-C cleaves only at lysine residues. One aliquot provides enough enzyme to digest ~5 µg protein.

Endoproteinase Glu-C: Solubilize 50 µg endoproteinase Glu-C (sequencing grade, Boehringer Mannheim) in 50 µl of Milli-Q water and let stand ~10 min. Transfer 5-µl aliquots (5 µg) into clean tubes, dry in a Speedvac evaporator, and store at

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–20°C (stable at least 1 month). Immediately before use, reconstitute enzyme in 50 µl Milli-Q water to obtain a 0.1 µg/µl working solution.

Endoproteinase Glu-C cleaves predominately at glutamic acid, but can sometimes cleave at aspartic acid. One aliquot provides enough enzyme to digest 50 µg of protein.

Clostripain: Solubilize 20 µg clostripain (sequencing grade, Promega) in 200 µl of manufacturer's supplied buffer to obtain a concentration of 0.1 µg/µl. Store enzyme solution at –20°C (stable up to 1 month).

Clostripain cleaves at arginine residues only. Enzyme solution provides enough enzyme to digest 50 µg of protein.

COMMENTARY

Background Information

The basic protocol described in this unit offers a simple method for obtaining amino-terminal and internal amino acid sequence data for PVDF membrane–bound proteins. The procedure is highly reproducible and is compatible with subsequent peptide mapping by reversed-phase HPLC (*UNIT 11.6*). Although proteins as large as 300 kDa have been successfully digested with this procedure, the average size of PVDF-bound proteins is ~80 kDa. The limits of the procedure appear to depend on the HPLC used for peptide isolation and the limitations of the protein sequencer.

Enzymatic digestion of a nitrocellulose-bound protein for internal sequence analysis was first reported by Aebersold et al. (1987), with a more detailed procedure later reported by Tempst et al. (1990). These procedures included treatment of the nitrocellulose-bound protein with PVP-40 (polyvinylpyrrolidone, M_r of 40,000) to prevent adsorption of the selected proteinase to any remaining nonspecific protein binding sites on the membrane. After extensive washing to remove excess PVP-40, the nitrocellulose-bound sample was cut into 1 × 1–mm pieces and then digested. Aebersold et al. (1987) digested the protein overnight at 37°C with either trypsin (in a buffer of 5% acetonitrile/100 mM Tris·Cl, pH 8.2) or V8 protease (in 5% acetonitrile/100 mM sodium phosphate), with no additional washing of the membrane after digestion. Tempst et al. (1990) digested the protein for 15 hr at 37°C with trypsin, endoproteinase Asp-N, endoproteinase Lys-C, chymotrypsin, and subtilisin (in 5% acetonitrile/100 mM NH_2HCO_3), followed by one wash with fresh digestion buffer. They also reported no success with endoproteinase Glu-C. Using the above procedures to digest PVDF-bound protein yielded poor results and generally required >25 µg of protein (Bauw et al., 1989; Aebersold, 1993).

Enzymatic digestion of both PVDF- and nitrocellulose-bound protein in the presence of 1% hydrogenated Triton X-100 (RTX-100) buffers (listed in Table 11.2.1) was first performed after treating the protein band with PVP-40, as described above (Fernandez et al., 1992). The RTX-100 buffer is a strong elution reagent that allows peptide recovery from the hydrophobic, high-binding-capacity PVDF membrane as well as from nitrocellulose. Unfortunately, the RTX-100 buffer also removes PVP-40 from the membrane, which can interfere with subsequent reversed-phase HPLC. Recent reports (Fernandez et al., 1994a,b) demonstrate that treatment with PVP-40 is unnecessary when RTX-100 is used in the digestion buffer. In addition to being a strong elution reagent, RTX-100 also acts as a blocking reagent, preventing proteinase adsorption to PVDF.

There are several clear advantages to using the RTX-100 digestion buffer procedure. First, it is applicable to PVDF (especially high-retention PVDF membranes such as ProBlott or Immobilon P^{Sq}). PVDF membranes are preferred over nitrocellulose because they allow higher recovery of peptides after digestion, result in fewer contaminants on subsequent HPLC analysis, and are compatible with other procedures used to analyze protein structure or amino acid analysis. The older procedures (Aebersold et al., 1987; Tempst et al., 1990) have not been successful with PVDF.

In addition, recovery of peptides from nitrocellulose is also higher when RTX-100 is used in the digestion buffer (Fernandez et al., 1992). The RTX-100 procedure does not require pretreatment of the protein band with PVP-40 and can be performed in a single step. Because this protocol does not require all the washes that the PVP-40 procedures do, there is less chance of protein loss during washing. Finally, this protocol requires considerably less

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time than the other procedures. Overall, it is the simplest and quickest method for obtaining quantitative recovery of peptides.

Critical Parameters

The largest source of sample loss is generally not proteinase digestion but the electroblotting of the sample. Electroblotting onto PVDF (preferably a high-binding type, such as ProBlott or Immobilon P^{sq}) rather than nitrocellulose is recommended because peptide recovery after digestion is usually higher from PVDF (Fernandez et al., 1992; Best et al., 1994). If nitrocellulose must be used because the protein is obtained already bound to nitrocellulose prior to digestion, never allow the membrane to dry out, as this will decrease peptide yields. Always electroblot the protein using a transfer tank system because yields from semi-dry systems are lower (Mozdzanowski and Speicher, 1990).

Using cleaner stains such as Ponceau S, amido black, or Coomassie brilliant blue-G with >90% dye content will increase detection of peptide fragments during reversed-phase HPLC. The key to the success of the procedure and quantitative recovery of peptides from both PVDF and nitrocellulose membranes is the use of RTX-100 in the buffer. This is desirable because non-hydrogenated Triton X-100 has several strong UV-absorbing contaminants (Fig. 11.2.1; Tiller et al., 1984). In addition, RTX-100 does not inhibit enzyme activity or interfere with peak resolution during HPLC as do ionic detergents such as SDS (Fernandez et al., 1992). Finally, the concentration of RTX-100 can be decreased to 0.1% with no loss in peptide yield (Fernandez et al., 1994b).

The addition of a second aliquot of enzyme after 4 to 6 hr initial digestion can improve peptide recovery (Best et al., 1994). A membrane should be cut into 1 × 1–mm pieces while keeping it wet to avoid buildup of static charge. These small pieces allow using the minimum amount of buffer to cover the membrane. The volume of digestion buffer used should be enough to cover the membrane (~50 µl) but can be increased or decreased depending on the amount of membrane present. The enzyme solution should be selected based on any additional knowledge of the protein available, such as its amino acid composition and whether it is basic or acidic. If the protein is a complete unknown, endoproteinase Lys-C or Glu-C would be a good choice. The enzyme-to-substrate ratio should be ~1:10; however, if the exact amount of protein is unknown, ratios of 1:2 through 1:50 are suitable for digestion and will not affect the quantitative recovery of peptides. After digestion, most of the peptides (~80%) are recovered in the original buffer and the additional washes are performed to ensure maximum recovery. Microbore reversed-phase HPLC is the best isolation procedure for peptides.

Troubleshooting

The greatest source of failure in obtaining internal sequence data is insufficient transfer of protein to the PVDF membrane, which leads to an inability to detect peptides during HPLC analysis. After staining the PVDF-bound protein, if the protein band cannot be detected by amido black staining but is observable with India ink (which is ~10-fold more sensitive), the protein quantity may be insufficient for this procedure. Similarly, if the protein band is detectable by radioactivity or immunostaining but not by protein stain, the quantity may be insufficient for subsequent HPLC analysis. Amino acid analysis (*UNIT 3.2*), amino-terminal sequence analysis, or at the very least, comparison with stained standard proteins on the blot, should be performed to help determine if enough material is present.

When a sufficient but small (less than 10 µg) amount of protein is available, problems may arise from misidentification of peptides on reversed-phase HPLC due to artifact peaks and contaminants. Although elimination of every contaminant is usually impossible, there are several strategic points and steps that can be taken to help reduce contamination. Simultaneous processing of a negative control (a protein-free segment excised from the PVDF membrane) will help to identify contaminants associated with the membrane and digestion buffer. The negative control must be processed through the same purification steps as the sample, including electroblotting and staining, and should be analyzed by HPLC immediately before or after the sample. A positive control (membrane-bound standard protein) is generally unnecessary but should be performed if the activity of the enzyme is in question or if a new lot number of enzyme is to be used.

Major sources of contaminants include the stains used to visualize the protein on the PVDF membrane, the microcentrifuge tubes used for digestion, reagents used during digestion and extraction of peptides, and the HPLC itself. Stains are the greatest source of contaminants, and Coomassie brilliant blue in particular frequently gives problems. Amido black and Pon-

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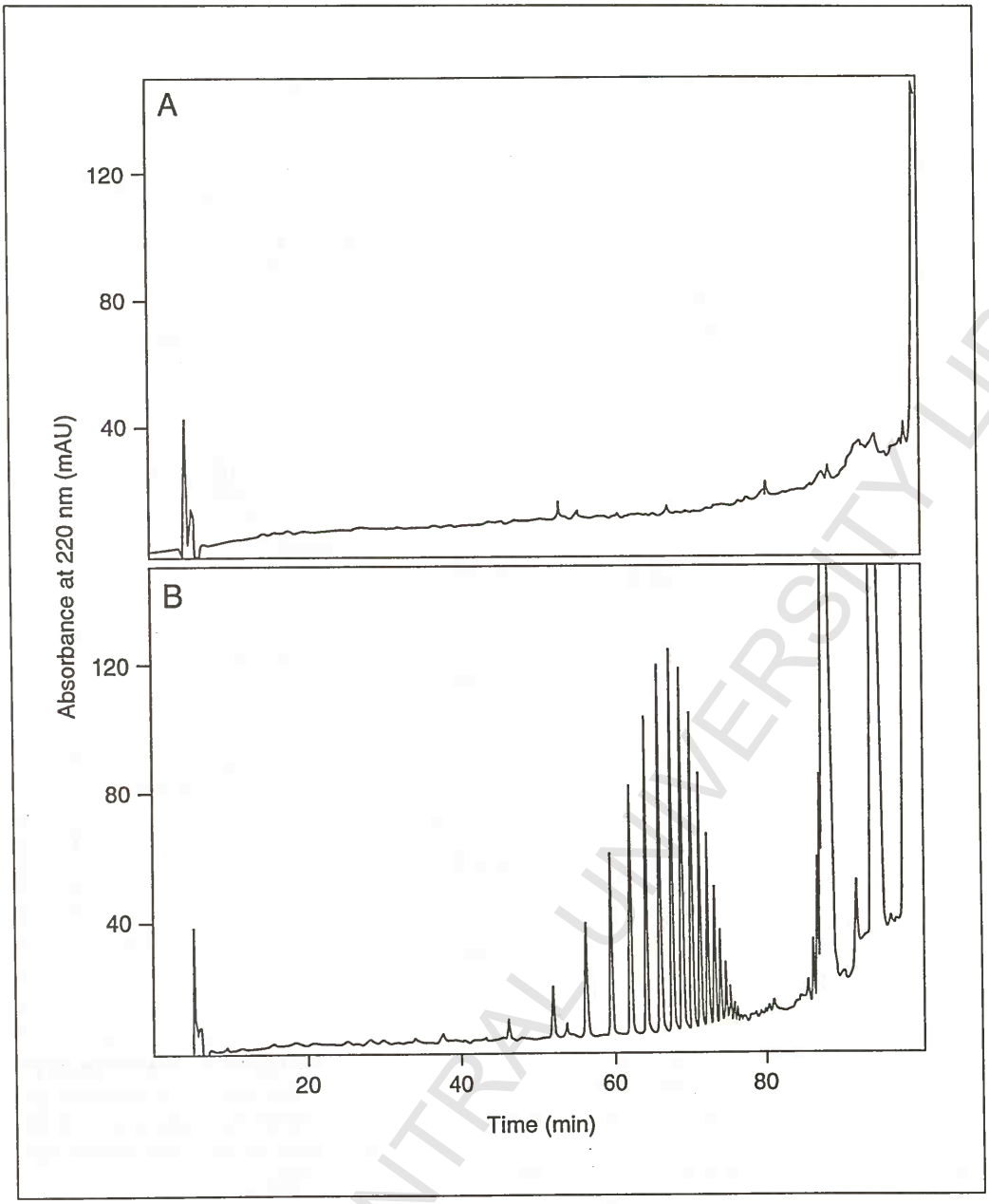

Figure 11.2.1 HPLC profiles of digestion buffer blanks. (**A**) Blank for 50 µl of 1% hydrogenated Triton X-100 (RTX-100)/10% acetonitrile/100 mM Tris·Cl, pH 8.0. (**B**) Blank for 1% Triton X-100/10% acetonitrile/100 mM Tris·Cl, pH 8.0. Both samples were incubated 20 hr at 37°C. Sample volumes were brought to 200 µl with 150 µl of 0.1% TFA and samples were analyzed on a Vydac C18 column (2.1 × 250–mm) using chromatographic conditions previously described (Fernandez et al., 1992). Peaks eluting at 50 to 100 min in panel B are UV-absorbing contaminants present only in Triton X-100.

ceau S are generally the cleanest, and Coomassie brilliant blue-G which has been chromatographically purified with a dye content >90% (e.g., Aldrich) appears to generate fewer contaminants than other less pure Coomassie brilliant blue stains. Surprisingly, microcentrifuge tubes can produce significant artifact peaks, which seem to vary with supplier and lot number. A blank containing only digestion buffer from a microcentrifuge tube should be included because some contaminants only appear after incubation in the RTX-100 buffer. The major concern with the digestion buffer is the hydrogenated Triton X-100 (see Figure 11.2.1), which is purchased as a 10% stock solution. Additional late-eluting peaks may be observed with certain lots of RTX-100, whereas other lots are completely free of UV-absorbing contaminants. Milli-Q water or water prepared as described by Atherton (1989)

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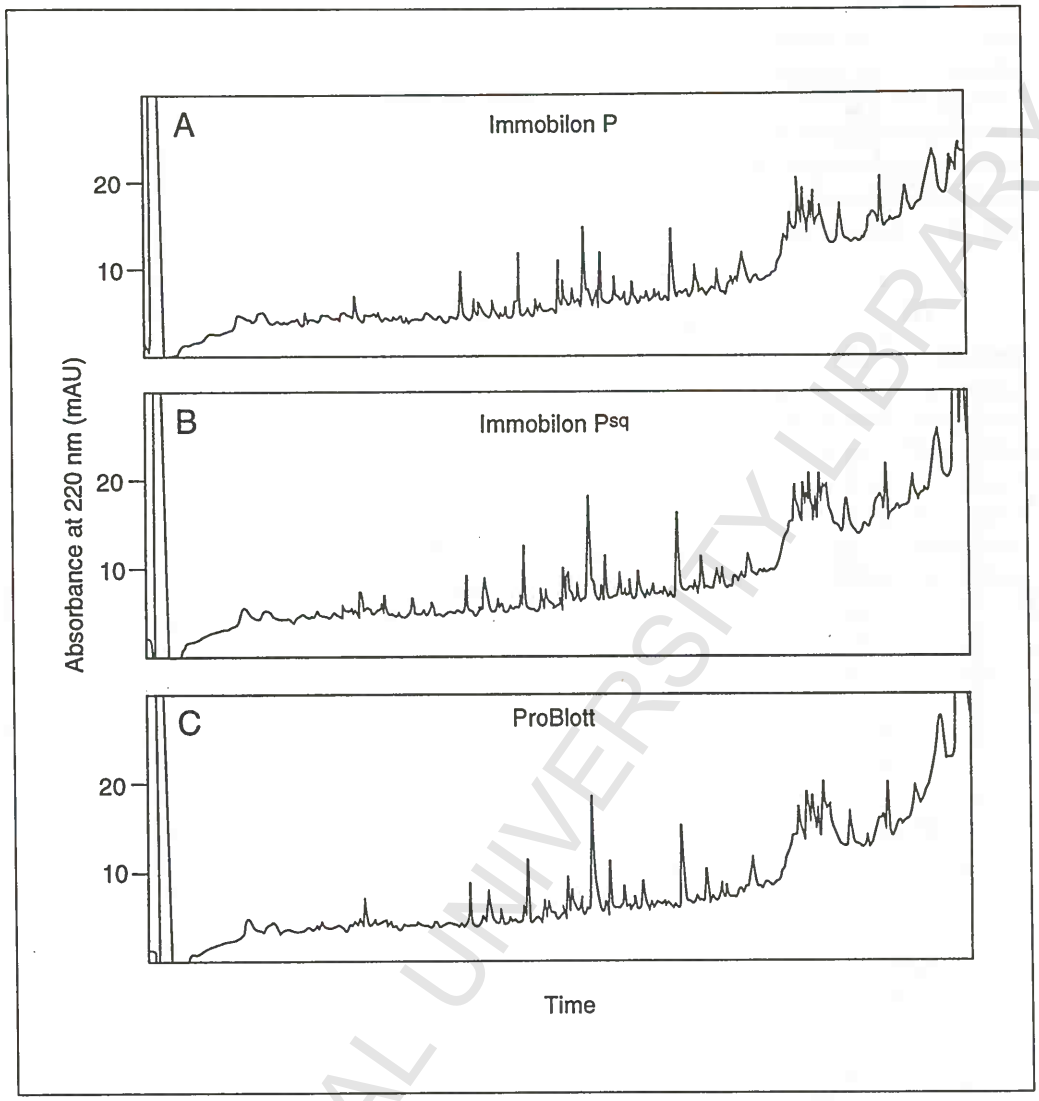

Figure 11.2.2 Peptide maps of trypsin digestion of human transferrin bound to different types of membrane. (**A**) Immobilon P; (**B**) Immobilon P^{sq}; and (**C**) ProBlott. Samples were prepared as described in the basic protocol. Four micrograms (~53 pmol) of transferrin was analyzed by SDS-PAGE, electroblotted to PVDF, and stained with Ponceau S prior to digestion. Chromatographic conditions were as previously described (Fernandez et al., 1992).

should be used for all solution preparation. An HPLC blank (i.e., a gradient run with no injection) should always be performed to determine which peaks are related to the HPLC.

As discussed in Critical Parameters, the concentration of RTX can be decreased without loss of peptide recovery. However, with a large amount of membrane this may not be the case. Previous procedures (Aebersold et al, 1987; Tempst et al., 1990; Bauw et al., 1989; Fernandez et al., 1992) required pretreatment of the membrane with PVP-40 to prevent any proteinase adsorption to the membrane. RTX-100 is essential for quantitative recovery of peptides from the membrane; however, RTX-100 also strips PVP-40 from the membrane, resulting in a broad, large, UV-absorbing contaminant that can interfere with peptide identification. The PVP-40 contaminant does not depend on the age or lot number of PVP-40; making fresh solutions does not prevent the problem (Aebersold, 1993). This appears to be more prevalent with nitrocellulose and higher-binding PVDF (ProBlott and Immobilon P^{sq}) than with lower-binding PVDF (Immobilon P), and depends on the amount of membrane used. The PVP-40 contaminant also appears to elute earlier in the chromatogram as the HPLC column ages, becoming more of a nuisance in visualizing peptides. Therefore, using PVP-40 to prevent enzyme adsorption to the membrane should be avoided.

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Peptide mapping by reversed-phase HPLC is described in detail in *UNIT 11.6*; however, there are a few considerations that are worth discussing here. A precolumn filter (Upchurch Scientific) must be used to prevent small membrane particles from reaching the HPLC column. Inspection of the pooled supernatants for visible pieces of PVDF can prevent clogs in the microbore tubing. Membrane fragments can be removed either with a clean probe (e.g., pointed tweezers, wire or thin pipet tip) or by spinning in a centrifuge and transferring the sample to clean vial.

Anticipated Results

Peptide mapping by reversed-phase HPLC after digestion of the membrane-bound protein should result in several peaks on the HPLC. Representative peptide maps from trypsin digestion of human transferrin bound to different PVDF membrane types—Immobilon P, Immobilon P^{sq}, and ProBlott—are shown in Figure 11.2.2. Peptide maps should be reproducible when performed under the same digestion and HPLC conditions as described in this unit, as demonstrated by Figure 11.2.2. In addition, the peptide maps from proteins digested on PVDF membranes are comparable if not identical to maps derived from proteins digested in solution, indicating that the same number of peptides are recovered from the membrane as from solution. The average peptide recovery is generally 40% to 70% based on the amount of protein analyzed by SDS-PAGE, and 70% to 100% based on the amount of protein bound to PVDF (as determined by amino acid analysis). Recovery of peptides from the membrane tends to be quantitative, and the greatest loss of sample seems to occur during electroblotting.

Time Considerations

The entire procedure can be done in ~24 hr plus the time required for peptide mapping by reversed-phase HPLC (see *UNIT 11.6*). Cutting the membrane takes ~10 min, incubation after the digestion buffer is added takes 5 to 30 min, digestion at 37°C takes 22 to 24 hr, and extraction of the peptides requires ~20 min.

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Key References

Fernandez et al., 1994a. See above.

Describes digestion with and without PVP-40 and applies it to unknown proteins.

Fernandez et al., 1994b. See above.

Describes digestion procedure and emphasizes applicability to different types of PVDF membranes and the concentration of RTX-100 buffer.

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Digestion of Proteins in Gels for Sequence Analysis

A high percentage of eukaroytic proteins have blocked amino termini, so it is usually necessary to cleave an "unknown" protein chemically or enzymatically to obtain the partial sequences needed for cDNA cloning. Because SDS-polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 10.1*) is the current method of choice for the final purification of the >25 pmol amounts of protein that are usually required for internal sequencing, procedures that can be used to digest proteins in situ in SDS-polyacrylamide gels are often the most useful and have the added benefit of eliminating losses that may occur during blotting. Two alternative strategies have been developed to respond to this need and to deal with the unique problems posed by SDS, which are that SDS inhibits trypsin, one of the enzymes that is most commonly used for internal sequencing studies, and also interferes with reversed-phase HPLC.

In the Basic and Alternate Protocol 2, SDS is removed from the gel prior to enzymatic cleavage by the staining and subsequent washing steps. The Basic Protocol calls for an acetonitrile wash to remove residual SDS from the protein sample. A different detergent, Tween 20, is then added back to the sample to maintain the solubility of the denatured protein. In Alternate Protocol 2, the gel slices are washed with ammonium bicarbonate and the protein samples digested in the absence of detergent.

Alternate Protocol 1 utilizes lysyl endopeptidase, an enzyme resistant to SDS; the digestion can therefore be carried out without prior removal of the SDS. Ultimately, SDS is removed via an anion-exchange precolumn that immediately precedes the reversed-phase HPLC column. In all cases, the peptides resulting from in-situ digestion are extracted from the gel matrix, then separated via reversed-phase HPLC prior to amino acid sequencing.

Before beginning the enzymatic digests described in these protocols, it is helpful to determine the amount of protein present in the sample via amino acid analysis, as described in Support Protocol 1. Reducing and alkylating proteins separated by SDS-PAGE, as described in Support Protocol 2, facilitates the identification of cysteine residues during subsequent peptide sequencing reactions.

DIGESTION OF PROTEINS IN GELS IN THE PRESENCE OF TWEEN 20

BASIC PROTOCOL

In this protocol, which is a slight modification of the Rosenfeld et al. (1992) procedure, an acetonitrile wash is used to remove residual SDS and Coomassie brilliant blue from the excised gel slice containing the protein of interest. The washed gel is then partially dried prior to rehydrating in the presence of the enzyme of choice—usually trypsin or lysyl endopeptidase—in a buffer containing Tween 20. The Tween 20 presumably helps both to remove residual SDS from the protein and to maintain the solubility of the denatured protein and its resulting cleavage fragments. After digestion, the peptides are alkylated with iodoacetic acid to facilitate identification of cysteine residues during amino acid sequencing (see Support Protocol 1).

After digestion, the peptides are extracted from the gel and separated on a C18 reversed-phase HPLC column. Refer to *UNIT 11.6* and to Stone et al. (1990, 1991, 1993) for further discussion of HPLC mapping and peptide isolation.

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Materials

Protein sample separated on SDS-polyacrylamide gel (*UNIT 10.1*; include appropriate standard protein on gel) and stained with Coomassie brilliant blue (*UNIT 10.5*)
50% (v/v) acetonitrile in 0.2 M ammonium carbonate, pH 8.9
0.02% (v/v) Tween 20 (Sigma) in 0.2 M ammonium carbonate, pH 8.9
0.1 mg/ml modified trypsin in manufacturer's dilution buffer (Promega; stable at least 2 years when stored at –20°C)
0.1 mg/ml lysyl endopeptidase (*Achromobacter* Protease I, Wako Chemicals USA) in 2 mM Tris·Cl, pH 8.0 (store <2 years at –20°C)
0.2 M ammonium carbonate, pH 8.9
0.1% (v/v) trifluoroacetic acid (TFA)/60% (v/v) acetonitrile
2 M urea/0.1 M NH_4HCO_3
Buffer A: 0.06% (v/v) TFA
Buffer B: 0.052% (v/v) TFA in 80% (v/v) acetonitrile

Water-bath sonicator
0.22 µm Ultrafree filter unit (Millipore)
HPLC system (Hewlett Packard 1090M or equivalent), equipped with 250-µl injection loop
Vydac C18 2.1 × 250–mm, 300-Å pore size, 5-µm particle size reversed-phase HPLC column (Separations Group) or equivalent
Fraction collector with peak separator (e.g., Model 2150; Isco)

Prepare the sample for digestion

1. Excise protein band of interest from stained gel. Also excise two control sections of gel: one containing protein standard (positive control), and one approximately equal in size to band of interest that does not contain any protein ("blank" negative control). Estimate the total volume of gel slices: total volume (mm^3) = length × width × gel thickness.

   Although more than one gel lane can be pooled for digestion, every effort should be made to keep the protein in as few lanes as possible and to attain the highest possible ratio of protein to gel volume.

   The blank control section of gel is useful for readily identifying artifact peaks in the final HPLC chromatogram that may arise from reagents, stains, or autolysis of the protease. For discussion of appropriate standard proteins for positive controls, see Critical Parameters and Troubleshooting.

2. Remove a portion of gel slice containing protein (i.e., 10% to 15%) and subject samples to amino acid analysis (see Support Protocol 1).

   Amino acid analysis is used to determine the amount of protein present in the sample that is to be digested. The gel slice should contain ≥25 pmol of protein and the minimum recommended protein density is 0.05 µg protein/mm^3 gel. If this analysis indicates that a sufficient amount of protein is present at a sufficiently high density, continue with step 3. Otherwise, additional protein should (if possible) be prepared and pooled with the sample.

3. Cut each gel slice (sample and control) into ~1 × 2–mm sections. Place each set of fragments in a 1.5-ml microcentrifuge tube and add ~150 µl of 50% acetonitrile in 0.2 M ammonium carbonate, pH 8.9.

   A sufficient volume should be added so that the sample can be shaken efficiently. If more than one lane of protein is being digested, an additional ~150 µl acetonitrile/ammonium carbonate may be added per lane and a total of about 8 lanes can fit reasonably well in a 1.5-ml microcentrifuge tube.

   From this step onward, process both controls in parallel with the sample containing the protein of interest.

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4. Place sample on a rocking table and incubate 20 min at room temperature.
5. Remove wash buffer and save in a clean 1.5-ml microcentrifuge tube.
6. Repeat wash with another 150 µl acetonitrile/ammonium carbonate.
7. Remove wash buffer and add to previous sample. Lyophilize gel pieces in a Speedvac evaporator until their volume is reduced by ~50%.

Digest the protein

8. Dilute enzyme of choice (modified trypsin or lysyl endopeptidase) to a concentration of 33 µg/ml using 0.02% Tween 20 in 0.2 M ammonium carbonate, pH 8.9. Add a volume of diluted enzyme approximately equal to original gel volume determined in step 1.

   Assume that 1 mm^3 = 1 µl.

9. If gel pieces are not completely immersed, add 0.02% Tween 20 in 0.2 M ammonium carbonate until pieces are covered with buffer.
10. Incubate samples 24 hr at 37°C.

Alkylate the digested sample

11. Add 50 µl of 0.2 M ammonium carbonate (pH 8.9) to each sample. If necessary, add additional 0.2 M ammonium carbonate until pieces are completely covered with buffer.

    This may be necessary because the gel swells during the 37°C incubation in step 10.

12. Reduce and alkylate sample (see Support Protocol 2).

Extract the peptides from the gel

13. Add 100 µl of 0.1% TFA/60% acetonitrile. If necessary, add additional TFA/acetonitrile until pieces are completely covered with buffer. Incubate 30 to 40 min on rocking table at room temperature.

    If a rocking table is not available, vortex the sample intermittently.

14. Sonicate 5 min in a water-bath sonicator.
15. Remove wash buffer (containing the extracted peptides) to a clean 1.5-ml microcentrifuge tube. Repeat extraction steps 13 and 14 with fresh TFA/acetonitrile.
16. Remove wash buffer and combine with buffer from first TFA/acetonitrile wash. Discard gel pieces. Add 100 µl of 2 M urea/0.1 M NH_4HCO_3 to combined wash buffer samples.
17. Lyophilize sample in a Speedvac evaporator until final volume is <100 µl.

    This will ensure sufficient removal of the CH_3CN so that the resulting peptides can be directly loaded on a reversed-phase HPLC column.

18. Dilute sample to 110 µl with HPLC-grade water.
19. Transfer sample to a 0.22-µm filter unit and microcentrifuge for 5 min at maximum speed.
20. Equilibrate Vydac C18 reversed-phase HPLC column at 0.15 ml/min in 98% buffer A/2% buffer B. Inject the filtrate from step 19 (containing eluted peptides) onto column.

    A Vydac C18 (2.1 × 250–mm, 300-Å pore size, 5-µm particle size) or comparable

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column is recommended for isolation of 25 to 250 pmol of digested peptides. Larger amounts may be separated on 4.6-mm columns (Stone et al., 1991).

21. Elute with buffer A/buffer B mixture according to the following gradient:

    0 to 60 min 2% to 37% buffer B
    60 to 90 min 37% to 75% buffer B
    90 to 105 min 75% to 98% buffer B.

22. Collect eluate in 1.5-ml capless microcentrifuge tubes placed in the top of 13 × 100–mm test tubes. Cap all sample tubes immediately following the completion of the collection run to prevent evaporation of the acetonitrile. Store eluted peptides at 5° to 10°C.

ALTERNATE PROTOCOL 1

DIGESTION OF PROTEINS IN GELS IN THE PRESENCE OF SODIUM DODECYL SULFATE

In this protocol, the gel slice containing the protein of interest is presoaked in a sodium dodecyl sulfate (SDS)/Tris buffer at pH 9.0. The slice is then digested in situ with lysyl endopeptidase (*Achromobacter* protease I) in the presence of SDS. After digestion, the gel pieces are crushed with a nylon mesh and the peptides extracted using an SDS/Tris buffer. The SDS is removed from the sample prior to reversed-phase separation of the peptides by a DEAE (or equivalent anion exchange) HPLC precolumn. The following protocol is a slight modification of the original procedure described by Kawasaki et al. (1990). In this modified protocol, the amount of protein present in the samples is determined by amino acid analysis and there is an additional carboxymethylation step at the end of the procedure.

Additional Materials (also see Basic Protocol)

0.1% (w/v) SDS in 100 mM Tris·Cl, pH 9.0
Nylon mesh (109 mesh)
DEAE HPLC precolumn or equivalent

Prepare the sample for digestion

1. Soak the stained and destained gel 1 hr in H_2O.

   This will reduce the concentrations of acetic acid and methanol in the gel.

2. Excise the protein band of interest from the gel, along with sections for positive and negative controls, and estimate protein concentration in protein band sample as in steps 1 and 2 of the Basic Protocol.

3. Cut each gel slice into ~1 × 2–mm pieces. Add ~100 µl of 0.1% SDS in 100 mM Tris·Cl (pH 9.0) to each sample. Preincubate sample 1 hr at 37°C.

   Try to keep volume of SDS to a minimum. Add only enough to cover gel pieces.

   From this step onward, process controls in parallel with the sample containing the protein of interest.

Digest the protein

4. Add a volume of lysyl endopeptidase that yields a 1:10 (w/w) ratio of enzyme to sample protein. Incubate 24 hr at 37°C.

   Dilutions of the 1 mg/ml lysyl endopeptidase stock should be made in 2 mM Tris·Cl, pH 8.0. According to the original protocol (Kawasaki et al., 1990), the final enzyme concentration must be >2 µg/ml. Therefore, if a 1:10 (w/w) ratio yields an enzyme

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concentration below this level, increase the ratio until this minimum concentration is reached.

Extract the peptides from the gel

5. Transfer supernatant to a clean 1.5-ml microcentrifuge tube.
6. Crush the gel pieces through a nylon mesh as follows: Cut a hole in the bottom of the filter unit section of a 0.22-µm filter. Replace filter with a 2-in. (5-cm) square of nylon mesh and place filter in a 1.5-ml microcentrifuge tube. Place gel pieces on top of nylon mesh. Microcentrifuge tube 10 to 15 min at maximum speed.
7. Remove filter from microcentrifuge tube. Add 500 µl of 0.1% SDS in 100 mM Tris·Cl (pH 9.0) to crushed gel pieces at bottom of tube and incubate with shaking 1 hr at room temperature.

   Shake sample on rocking table or vortex intermittently.

8. Transfer supernatant to a 0.22-µm filter unit and microcentrifuge 10 to 15 min at maximum speed.
9. Combine supernatant with sample from step 5.
10. Lyophilize sample in a Speedvac evaporator until volume is <200 µl.
11. Dilute sample to 210 µl with water.

Alkylate the digested protein

12. Reduce and alkylate the protein (see Support Protocol 2).
13. Separate alkylated sample on 2.1 × 250–mm Vydac C18 reversed-phase HPLC column that is equipped with a DEAE precolumn as in steps 20 to 22 of the Basic Protocol.

DIGESTION OF PROTEINS IN GELS IN THE ABSENCE OF DETERGENT

ALTERNATE PROTOCOL 2

In this protocol, enzyme digestion is carried out in the absence of detergent. The protein is separated by SDS-PAGE and washed extensively with 0.2 M ammonium bicarbonate. After washing, the protein is reduced, alkylated, and digested with either modified trypsin or lysyl endopeptidase. The resulting peptides are then eluted from the gel using passive elution in 2 M urea/0.1 M ammonium bicarbonate prior to separation by reversed-phase HPLC.

***Additional Materials** (also see Basic Protocol)*

0.2 M ammonium bicarbonate
2 M urea/0.1 M ammonium bicarbonate

Prepare the sample for digestion

1. Excise the protein of interest from the gel, along with sections for positive and negative controls, and determine the protein concentration as in steps 1 and 2 of the Basic Protocol.
2. Cut each gel slice into 1 × 2–mm pieces and add 500 µl of 0.2 M ammonium bicarbonate to each sample.

   Gel pieces should be totally covered with 0.2 M ammonium bicarbonate to enable efficient shaking. If gel pieces are not totally immersed, add extra ammonium bicarbonate to completely cover sample.

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From this step onward, process controls in parallel with the sample containing the protein of interest.

3. Incubate sample on rocking table 30 min to 1 hr at room temperature.

   If rocking table is not available, vortex samples intermittently.

4. Transfer wash buffer to clean 1.5-ml microcentrifuge tubes and measure pH.

5. If pH is acidic, add an additional 500 µl of 0.2 M ammonium bicarbonate to gel pieces and incubate 2 hr at room temperature. Check the pH again after 2 hr. If pH of wash buffer is still acidic, repeat wash again; otherwise proceed to step 6.

6. Add sufficient volume of 0.2 M ammonium bicarbonate to gel pieces to completely immerse sample.

Alkylate the protein

7. Reduce and alkylate protein sample (see Support Protocol 2).

Digest the protein

8. Add an appropriate volume of enzyme to the alkylated sample to obtain a final enzyme concentration of 6 µg/ml for modified trypsin or 12 µg/ml for lysyl endopeptidase. Incubate 24 hr at 37°C.

9. Add 500 µl of 2 M urea/0.1 M ammonium bicarbonate to each sample. Incubate on a rocking table 6 hr at room temperature to extract peptides.

   If a rocking table is unavailable, vortex samples intermittently.

10. Transfer supernatant to a clean 1.5-ml microcentrifuge tube and freeze.

11. Add 500 µl of 0.2 M ammonium bicarbonate to gel pieces. Incubate on rocking table overnight at room temperature.

12. Combine this extract with supernatant from step 10. Lyophilize sample in a Speedvac evaporator until the volume is <200 µl.

13. Adjust volume to 210 µl with water.

14. Transfer supernatant to a 0.22-µm filter unit and microcentrifuge 10 min at maximum speed.

15. Subject the filtrate containing eluted peptides to HPLC as in steps 20 to 22 of the Basic Protocol.

SUPPORT PROTOCOL 1

AMINO ACID ANALYSIS OF PROTEINS SEPARATED BY SDS-PAGE

Prior to beginning the enzymatic digests described in the basic and alternate protocols, an amino acid analysis should be carried out on 10% to 15% of the gel slice to accurately determine the amount of protein that is present.

Materials

Gel slice (Basic Protocol, Alternate Protocol 1, or Alternate Protocol 2)
0.2% (v/v) phenol in 6 N HCl, containing 2 nmol/100 µl norleucine internal standard

10 × 75-mm Pyrex test tube
Amino acid analyzer (Beckman 7300 or equivalent)

1. Add 100 µl of 0.2% phenol in 6 N HCl (with 2 nmol/100 µl norleucine) to gel slice. Hydrolyze sample 16 to 24 hr at 115°C.

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2. After hydrolysis, transfer supernatant to a 10 × 75-mm Pyrex test tube and lyophilize to dryness using a Speedvac evaporator.
3. Dissolve hydrolysate in buffer recommended for the amino acid analyzer. Load sample onto analyzer.
4. Determine protein concentration as described in *UNIT 3.2*. Continue with digestion as described in the Basic Protocol, Alternate Protocol 1, or Alternate Protocol 2.

REDUCTION AND ALKYLATION OF PROTEINS

SUPPORT PROTOCOL 2

The major benefit of reducing and alkylating proteins separated by SDS-PAGE is that it facilitates the subsequent identification of cysteine residues via Edman degradation. Reduction and alkylation convert cysteine to a more stable phenylthiohydantoin (PTH) derivative.

Materials

Gel pieces in buffer (basic or alternate protocols)
45 mM dithiothreitol (DTT)
100 mM iodoacetic acid (IAA)
50°C incubator

1. Estimate the total volume of sample (gel plus buffer).

   Sample volumes can be most easily estimated by comparing to a "standard" set of microcentrifuge tubes containing known volumes of water.

2. Calculate volume of DTT ("*x*") needed to yield a final DTT concentration of 1 mM: $(x) \times (0.045\ M\ stock) = (total\ volume) \times (0.001\ M)$.
3. Add DTT and incubate sample 20 min at 50°C.
4. Remove sample from incubator and let cool to room temperature. Add a volume of 100 mM IAA equal to the volume (*x*) of DTT added in step 3.
5. Incubate sample 20 min at room temperature in the dark, to prevent formation of iodine which could react with tyrosine residues.
6. Continue with protein digestion (see Basic Protocol, step 13; Alternate Protocol 1, step 13; or Alternate Protocol 2, step 8).

COMMENTARY

Background Information

Because a high percentage of eukaryotic proteins have blocked amino termini (Brown and Roberts, 1976; Dreissen et al., 1985), direct amino terminal sequencing often ends in failure. In fact, when the amount of a eukaryotic protein is extremely limiting (i.e., <50 pmol), it is usually far better to assume the amino terminus is blocked and proceed directly with enzymatic or chemical cleavage. Because SDS-PAGE is often the last step in protein purification, cleavage protocols that can be directly carried out on proteins in SDS polyacrylamide gels are often the most useful, as they avoid the losses that may occur during elution of the proteins from the gels or during electroblotting of proteins onto membranes.

Trypsin and lysyl endopeptidase are often the preferred enzymes for cleaving proteins in SDS-polyacrylamide gels. These enzymes have high specificity and produce peptides of a size that separate optimally via reversed-phase HPLC (typically 5 to 25 residues). Based on the average occurrence of lysine (5.7%) and arginine (5.4%) in proteins listed in the Protein Identification Resource Database, digestion with either of these enzymes would be expected to produce peptides with an average length of 17 residues (for lysyl endopeptidase) or ~9 residues (for trypsin).

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Enzymatic digestions of proteins in gels have been carried out both in the absence (Stone and Williams, 1993; Ward et al., 1990) and the presence of detergents such as SDS (Kawasaki et al., 1990) or Tween 20 (Rosenfeld et al., 1992). In all instances, the protease is allowed to diffuse into the gel and the resulting peptides are subsequently obtained by diffusion out of the gel.

The Basic Protocol is a slightly modified version of the Rosenfeld et al. (1992) procedure in which the SDS is removed from the gel slice (prior to carrying out the digest) by two quick washes with 50% acetonitrile/0.2 M ammonium carbonate (pH 8.9). After removal of the SDS, the gel slices are dried to half their original volume and then partially rehydrated in a 200 mM ammonium carbonate (pH 8.9) buffer containing Tween 20 and the enzyme.

It is possible to digest proteins in the presence of SDS by using lysyl endopeptidase, an enzyme that is active in the presence of SDS, and by using an anion-exchange precolumn to remove the SDS immediately prior to reversed-phase HPLC (Kawasaki and Suzuki, 1990). This modified version of a procedure described by Kawasaki et al. (1990) is presented in Alternate Protocol 1. In this procedure, the SDS binds to the precolumn and the peptides pass through to the reversed-phase HPLC column. Although the precolumn is regenerated by the high concentration of acetonitrile present at the end of the gradient, in practice, the precolumn should be replaced at least every five runs or after a total of 1 mg SDS has been applied to the column (Williams et al., 1993).

There is currently a lack of definitive data regarding which of the three procedures described in this unit provides the highest yield of peptides from a given amount of protein separated on an SDS-polyacrylamide gel (Williams et al., 1993). The procedure described in the Basic Protocol is preferred because it is the simplest and can be used for a wide range of proteases, including trypsin, lysyl endopeptidase, chymotrypsin, and endoproteinase Glu-C. It was observed that one sample that was resistant to digestion in the absence of a detergent, as described in Alternate Protocol 2, was successfully digested using the procedure described in the Basic Protocol, perhaps due to the presence of Tween 20 (K.L.S. and K.R.W., unpub. observ.).

Critical Parameters and Troubleshooting

Two of the most critical questions that need to be addressed prior to digesting a protein in a polyacrylamide gel are whether or not the protein is pure and whether or not the Coomassie blue–stained band that has been selected is actually responsible for the activity of interest.

The first question can often be addressed by subjecting a small aliquot of the protein to two-dimensional gel electrophoresis (*UNIT 10.4*), where the first direction is an isoelectric focusing step. Keep in mind, however, that although the appearance of a single Coomassie blue–stained spot following two-dimensional electrophoresis provides good evidence for homogeneity, microheterogeneity with respect to the isoelectric point may result from post-translational modifications, such as phosphorylation, which would have little impact on internal amino acid sequence.

To ensure that the protein band selected is responsible for the activity of interest, aliquots of fractions eluted from the last chromatography step can be subjected to another round of SDS-PAGE. It should be possible to directly correlate the protein's activity with the intensity of the candidate band on SDS-PAGE and to thus confirm the identity and purity of this band.

There are several other approaches that may be taken to ensure that the appropriate band has been selected for internal amino acid sequencing. Gel filtration can be used to independently verify the approximate molecular weight of the protein of interest. A protein separated by SDS-PAGE often can be renatured to confirm its activity. In the case of nucleic acid binding proteins, the protein preparation can be photocrosslinked to a ^{32}P-labeled nucleic acid target site. When the nucleic acid is degraded with a nuclease, if the candidate band is the correct DNA-binding protein, it should be labeled with ^{32}P (Safer et al., 1988).

Once the identity of the Coomassie blue–stained protein band has been confirmed, it is important to determine whether a sufficient amount of protein is present for the experiment to proceed. Investigators who rely on relative Coomassie blue staining and colorimetric assays often overestimate the amount of protein present (sometimes by as much as 5- to 10-fold). To obtain an accurate estimate of protein concentration, an aliquot of the Coomassie blue–stained gel band should be subjected to hydrolysis and ion-exchange amino acid analysis as described in Support Protocol 1. In the case of 50 pmol of a 50 kDa protein, 15% of the band (~0.4 μg of protein) would be a rea-

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sonable fraction of the sample to commit to this step.

Although glycine, cysteine, tryptophan, and sometimes histidine and arginine cannot be quantified by this analysis, it nonetheless provides a more accurate estimate of the amount of protein than does Coomassie blue staining. The latter approach can be affected by how well different proteins stain with Coomassie brilliant blue.

Because the "background" obtained from hydrolyzing and analyzing blank sections of SDS-polyacrylamide gels averages ~0.09 μg (Williams et al., 1993), an aliquot of a control section of gel that appears not to contain any protein should also be hydrolyzed along with the slices containing the protein of interest. The blank values should be subtracted from those obtained for the unknown protein sample. Although the Basic Protocol has been used successfully on as little as 16 pmol of transferrin and 26 pmol of an unknown protein, a minimum of 75 pmol (as determined by hydrolysis/amino acid analysis) should be used both to ensure a high probability of success and to improve the quality and length of the resulting sequence data.

A final parameter to consider is the "density" (i.e., in $\mu g/mm^3$) of the protein in the gel band. All five of the unsuccessful digests listed in Table 11.3.1 contained proteins at densities ≤ 0.05 $\mu g/mm^3$ (with a mean density for these five samples of 0.03 $\mu g/mm^3$). Because the group of unsuccessful samples included two samples containing 101 to 200 pmol of protein and two samples with >200 pmol of protein, it appears that, within reasonable limits, the density of the protein in the gel band is a more important determinant of success than is the absolute amount of protein. Although samples with densities as low as 0.02 have been digested successfully, to ensure success, samples should have a minimum density of ≥ 0.05 $\mu g/mm^3$. Obviously, everything possible should be done to ensure that the sample is loaded in one or two lanes as opposed to three or more. Typically, gels that are ≤0.75 mm in thickness should be used and a minimum of 1 μg of the protein of interest should be included in each lane.

If a minimum of 25 pmol protein (at a density > 0.05 $\mu g/mm^3$) is digested, the success rate should be well above 90% (see Table 11.3.1). The laboratory carrying out the work should be accustomed to routinely isolating <10 pmol amounts of peptides and to sequencing in the several hundred femtomole range.

Because human transferrin (M_r = 75,000) digests as well or better using the Basic Protocol than does any other "standard" protein, it serves as an excellent positive control for proteins in the >50 kDa range. For proteins <50 kDa, carbonic anhydrase (M_r = 29,000) provides a reliable positive control. The positive control is just as important as the negative control (i.e., the blank section of gel that does not contain any protein). Hence, positive and negative controls should be performed along with all samples subjected to enzymatic digestion in a gel. In other words, a similar amount of transferrin should be run on the same gel as the sample and should be digested, carboxymethylated, and subjected to analytical HPLC along with the sample. Obviously, if neither the transferrin nor the sample digests, the fault does not lie with the sample.

If the above criteria have been met and there is no trivial reason (e.g., failure to add the enzyme solution to the sample) for the failure of a sample to digest, then a lack of digestion may reflect the intrinsic resistance of the sample to enzymatic cleavage. A common reason for this is extensive glycosylation. Although <10% (by weight) carbohydrate does not seem to adversely affect cleavage (e.g., of transferrin), high levels of glycosylation may well prevent or severely limit cleavage. If the extent of glycosylation is significantly above 10%, the carbohydrate should be enzymatically removed immediately prior to SDS-PAGE.

Finally, even after complete deglycosylation, a few proteins, such as the major subunit of the Duffy blood group system (Chaudhuri et al., 1993), may be totally resistant to digestion in gels by trypsin, lysyl endopeptidase, or chymotrypsin (with the latter enzyme so far uniformly failing to cleave any protein that was resistant to trypsin). In this instance, cyanogen bromide (CNBr) can be used to chemically digest the protein sample (see *UNIT 11.4*). Assuming that SDS-PAGE is a necessary final purification step, CNBr cleavage has to be carried out in the gel (Jahnen et al., 1990).

Because CNBr-generated peptides do not usually separate well by reversed-phase HPLC, the resulting peptides should be eluted from the gel and then separated via subsequent SDS-PAGE. The separated peptides can then be electroblotted onto a high-capacity PVDF membrane (*UNIT 10.7*), stained (*UNIT 10.8*), and sequenced. Alternatively, CNBr cleavage could be carried out prior to the Basic Protocol. Of course, prior CNBr cleavage will make at

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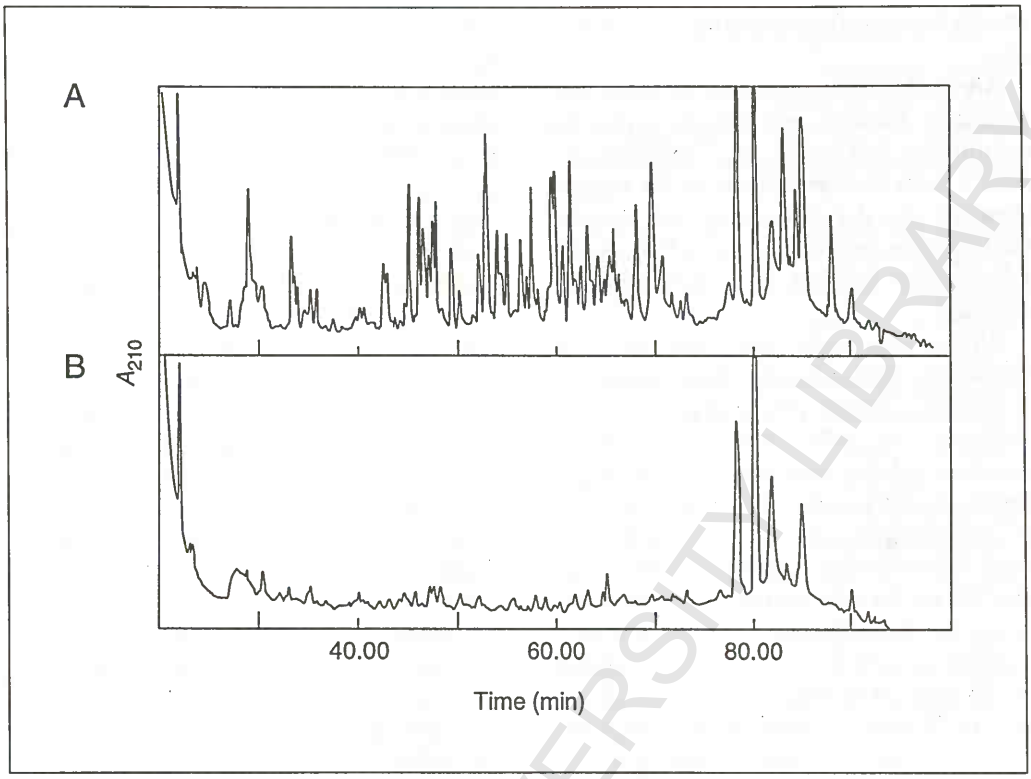

Figure 11.3.1 (**A**)HPLC profile of tryptic digestion of 42 pmol of transferrin. Protein was separated on a 12.5% SDS-polyacrylamide gel and digested as described in the Basic Protocol. Following trypsin digestion, the resulting peptides were fractionated on a Vydac C18 column via reversed-phase HPLC as described in the Basic Protocol. The Y-axis is absorbance at 210 nm (full scale = 0.02) and the X-axis is in terms of minutes. (**B**) Analytical HPLC profile for a control "digest" that was carried out on a section of gel that appeared not to contain any protein.

least some regions of the protein susceptible to further cleavage via proteolysis.

It is important to point out that, based on several hundred proteins subjected to analysis in the Keck Facility over the last few years, <2% of proteins fall into this intractable category (K.L.S. and K.R.W., unpub. observ.). Hence, assuming that the protein is not extensively glycosylated, resistance of the protein to digestion should be one of the last rather than one of the first reasons advanced to account for a failed digest.

Anticipated Results

Figure 11.3.1 shows the HPLC profile from a tryptic digestion (following the Basic Protocol) of 42 pmol transferrin that was separated in a 12.5% SDS-polyacrylamide gel at a protein/gel-volume density of 0.19 μg/mm^3. By comparing the transferrin sample (A) to the "blank" chromatogram (B), it is possible to quickly identify most of the artifact absorbance peaks that arise from trypsin autolysis products, reagents, and Coomassie blue (which typically gives rise to one or more peaks eluting after ~80 min).

Table 11.3.1 summarizes the results for 53 proteins digested according to the Basic Protocol or Alternate Protocol 2 (in the absence of detergent). One of the first conclusions that can be drawn from this data is that the overall success rate of these two procedures (even when some of the samples do not meet the criteria given above) appears to be at least 90%. Another conclusion is that the major benefit of going from the <50 pmol to the >200 pmol level is a progressive increase in the percent yield of an average peptide (i.e., from <6% to >14%), which directly translates into a larger number of positively identified residues for each peptide sequenced (i.e., from <9 to >14).

It should be noted that because the percent initial yields (i.e., overall recovery of peptide from a given amount of protein that is digested) are based on the initial sequencing yield, which is typically equal to only ~50% to 75% of the actual amount of peptide loaded, the actual recovery of tryptic peptides is probably from

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Table 11.3.1 Effect of Sample Amount on Digestion of Proteins in Gels[a]

Parameter	Amount of protein analyzed							
	<50 pmol		51-100 pmol		101-200 pmol		>200 pmol	
	Range	Mean or number	Range	Mean or number	Range	Mean or number	Range	Mean or number
Number of proteins		5		8		20		20
Mass of protein (kDa)	15-106	69	40-120	72	18-285	71	17-285	58
Amount of protein digested (pmol)	26-49	39	54-100	80	115-181	139	204-850	364
Density of protein bank ($\mu g/mm^3$)	0.03-0.12	0.07	0.02-0.09	0.05	0.02-0.31	0.09	0.05-0.66	0.21
% Initial yield[b]	2-11.1	5.7	1-37.6	10.6	0.6-68.9	11.7	0.005-50.5	14.4
Results of Sequencing								
# of Peaks sequenced per protein		1.4		2.0		2.1		2.5
≥4 Residues called (%)		88		76		76		80
Mixture (%)		12		0		12		2
No sequence obtained (%)		0		24		12		18
# of Residues called per peptide sequenced		8.71		10.6		12.0		14.2
Overall success rate[c] (%)		100		88		90		90

[a]Represents the analysis of 53 recent in-gel digests carried out in the W.M. Keck Facility at Yale University.

[b]Calculated as the yield of Pth-amino acid sequencing at the first or second cycle compared to the amount of protein that was digested as determined by hydrolysis and amino acid analysis of a 10% to 15% aliquot of the gel slice. Because initial yields of purified peptides directly applied onto the sequencer are usually about 50%, the actual % recovery of peptides from the above digests is probably twice the % initial yields given above.

[c]As judged by the ability to positively identify the sequence of at least 15 residues in one or two peptides or to obtain sufficient sequence to positively identify the protein via database searches. 34 of the above 53 proteins (64%) were identified based on these database searches.

1.3- to 2-fold above that given in Table 11.3.1 (i.e., it probably ranges from ~12% greater recovery at the <50 pmol level to >28% at the >200 pmol level). These average recoveries are particularly useful for judging whether the peptide sequenced was derived from the major component in the gel band that was digested. For instance, if the initial yield of a peptide from a digest of 350 pmol of protein in a gel is only 1%, it raises the possibility this peptide may have derived from a 10% contaminant.

Another conclusion from Table 11.3.1 is that, on average, >80% of peptides selected for sequencing provide usable sequences, as assessed by their symmetrical absorbance profile and laser desorption mass spectra (LDMS). The remaining 20% of samples either fail to sequence, contain mixtures of peptides, or turn out to be derived from autolysis of the protease. Occasionally, an autolysis product observed in the sample will not be present in the blank.

Without the benefit of LDMS, the fraction of symmetrical HPLC peptide peaks that provide usable sequences decreases to ~67% (Stone et al., 1992). When the criteria for selecting candidate peaks for sequencing are increased to include both the appearance of a symmetrical HPLC peak and a major/minor mass spectrometric peak ratio of at least 10 as judged by LDMS, the probability of obtaining useful data from sequencing any given HPLC absorbance peak increases to 90% (based on the last 200 peptides sequenced in the Keck Facility at Yale University; K.L.S. and K.R.W., unpub. observ.).

Obviously, because the sensitivity of LDMS is such that spectra can be routinely obtained on 25 to 500 fmol amounts of most tryptic peptides, this technique provides an extremely valuable adjunct to internal sequencing. In addition to providing an independent measure of peak purity, LDMS readily identifies Coo-

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massie blue, protease autolysis products, and other reagent peaks. In addition, the resulting peptide mass can be used to confirm the results of Edman degradation as well as to detect and often identify post-translational modifications such as phosphorylation.

Finally, the high percentage of proteins that are identified based on database searching of internal peptide sequences (64% based on the 53 proteins listed in Table 11.3.1) points out the absolute necessity of these searches. Unfortunately, in many instances these searches demonstrate that the major component in the Coomassie blue–stained band sequenced is not the protein of interest. Some of the proteins that have recently been identified via these searches (and which were obviously not responsible for the activity being purified) include: nucleolin, lactotransferrin, keratin, human serum albumin, vinculin, ubiquitin, collagen, alcohol dehydrogenase, tubulin, and—particularly when the purification involves immunoaffinity chromatography—immunoglobulins (K.L.S. and K.R.W., unpub. observ.). Because as few as 5 to 6 residues of sequence are often sufficient to identify a protein via a database search, the search can sometimes be performed while the peptide is actually being sequenced. In this way, sequencing can be terminated if the peptide is found to derive from a known protein.

Time Considerations

Enzymatic digestions of proteins in gels carried out in the presence of Tween 20 or SDS can be completed in 24 hr. Alternate Protocol 2 (carried out in the absence of detergent) requires 3 to 4 days to complete. The amino acid analysis will delay the start of the digestion for 2 to 3 days, but it is well worth the extra time, because proceeding with 50 pmol instead of 10 pmol may well mean the difference between success and failure. Isolating the peptide fragments via reversed-phase HPLC requires only a few hours and subsequent peptide sequencing requires 1 to 2 days.

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Chemical Cleavage of Proteins in Solution

Detailed structural analysis of proteins frequently involves digestion of samples (enzymatically or chemically) into smaller fragments. Enzymatic digestion (*UNIT 11.1*) generally produces a mixture of a large number of peptides, which require additional purification (e.g., by HPLC) before they can be analyzed further. Purification becomes more challenging when certain proteases, such as trypsin and *Staphylococcus aureus* V8, are used, owing to the high proportion of these recognition site residues in proteins.

Chemical cleavage, in contrast to enzymatic cleavage, usually targets residues and specific dipeptide linkages that occur at low frequencies in proteins. Thus, on average, fewer fragments will be generated and these fragments will be larger in size than those produced by standard enzymatic treatment. Consequently, in addition to reversed-phase, ion-exchange (*UNIT 8.2*), and size-exclusion (*UNIT 8.3*) chromatographies, analytical high-resolution electrophoresis (*UNIT 10.1*) can be used to purify resulting protein fragments.

In this unit, the four basic protocols present widely used reactions that are specific and efficient for the chemical cleavage of proteins in solution. Cyanogen bromide (CNBr; Basic Protocol 1) cleaves at methionine (Met) residues; 2-(2′-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine (BNPS-skatole; Basic Protocol 2) cleaves at tryptophan (Trp) residues; formic acid (Basic Protocol 3) cleaves at aspartic acid–proline (Asp-Pro) peptide bonds; and hydroxylamine (Basic Protocol 4) cleaves at asparagine-glycine (Asn-Gly) peptide bonds. Because Met and Trp residues are relatively low in abundance in most proteins, digestion with CNBr or BNPS-skatole will yield relatively long peptides. In general, reactions are chosen based on a priori knowledge of a protein's target residues. If no information is available, perform the reactions in the order listed.

Solid-phase digestions can be performed using similar chemical reactions to cleave proteins attached to membranes (*UNIT 11.5*).

CAUTION: Most chemicals used in the following protocols are toxic. Therefore, it is imperative that good laboratory practice be followed. All procedures and incubations should be performed in a well-ventilated chemical fume hood and laboratory personnel should wear appropriate protective laboratory clothing, including safety glasses and gloves. All chemical waste should be properly disposed of according to local regulations.

CLEAVAGE AT THE C-TERMINUS OF MET RESIDUES WITH CNBr

BASIC PROTOCOL 1

Cyanogen bromide (CNBr) cleaves proteins on the C-terminal side of unoxidized methionine (Met) residues. For proteins with multiple Met residues, fragments generated from this reaction can be incomplete (i.e., contain an internal unmodified Met or homoserine). After digestion with CNBr, resulting peptide fragments are fractionated by HPLC or electrophoresis prior to further characterization. This protocol also provides instructions for neutralizing CNBr, which is extremely toxic, after use.

Materials

Lyophilized protein sample in 1.5-ml capped polypropylene microcentrifuge tube
5 mg/ml CNBr in formic acid (see recipe)
10 M NaOH
Milli-Q water or equivalent

Aluminum foil or opaque container
5-ml glass or polypropylene tube

Additional reagents and equipment for reversed-phase (*UNIT 11.6*) or size-exclusion chromatography (*UNIT 8.3*), electrophoresis (*UNIT 10.1*), or electroblotting to PVDF membranes (*UNIT 10.7*)

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1. Add CNBr/formic acid to lyophilized sample containing 1 to 50 µg total protein to yield a final protein concentration of 1 µg/µl.

   The quantity of CNBr used for chemical cleavage of proteins at Met residues is based on a molar ratio of 200:1 (CNBr:Met). This ratio can be altered if the protein contains >2.3% Met residues.

2. Cap sample and vortex until protein is completely solubilized in the CNBr solution. Cover sample tube with aluminum foil or place in an opaque container. Incubate 24 hr at room temperature in a chemical fume hood.

   IMPORTANT NOTE: *This reaction must be performed in complete darkness to minimize unwanted side reactions with other amino acid side-chains.*

3. Neutralize unused CNBr by transferring remaining CNBr slowly to 5-ml tube containing 5 to 10 vol of 10 M NaOH. Rinse the pipet with 10 M NaOH before discarding.

   CAUTION: *Reaction of CNBr with NaOH is exothermic, generating heat and bumping of the solution. Therefore, add the CNBr solution slowly to minimize violent reactions. Once the CNBr is neutralized, it can be discarded according to halogen chemical waste disposal procedures.*

4. Add 10 vol Milli-Q water to sample tube. Lyophilize overnight or dry sample completely (1 to 2 hr) in a Speedvac evaporator.

   CAUTION: *After lyophilization or drying, defrost the cold trap and neutralize its contents by adding 10 M NaOH until the the pH reaches ≥7.0. Discard contents according to halogen chemical waste disposal procedures.*

5. Resolubilize sample in appropriate buffer for separation by reversed-phase (*UNIT 10.6*) or size-exclusion chromatography (*UNIT 8.3*) or for analysis by electrophoresis (*UNIT 10.1*) followed by electroblotting to PVDF membranes (*UNIT 10.7*).

   Some cleavage products can be difficult to resolubilize unless chaotropic agents (e.g., 6 M guanidine hydrochloride or 8 M urea) or detergents (e.g., 1% SDS or 1% cetyltrimethylammonium bromide) are used (see UNIT 1.3 & UNIT 6.1). Sonication is helpful for resolubilization, as is mild heat (i.e., in a 37°C water bath). Use of such agents may necessarily dictate the method chosen for fractionation. For example, detergent-treated samples are best analyzed by electrophoresis, whereas column chromatography should be used for guanidine- or urea-treated samples. Tris-tricine gels are recommended for electrophoretic separation of peptide fragments <10 kDa (UNIT 10.1).

BASIC PROTOCOL 2

CLEAVAGE AT THE C-TERMINUS OF TRP RESIDUES WITH BNPS-SKATOLE

BNPS-skatole, or 2-(2′-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine, cleaves proteins on the C-terminal side of unoxidized tryptophan (Trp) residues. For proteins with multiple Trp residues, fragments generated from this reaction can be incomplete (i.e., contain an internal unmodified or oxidized Trp). After digestion with BNPS-skatole, the resulting peptide fragments are fractionated by HPLC or electrophoresis prior to further characterization.

Materials

Lyophilized protein sample
Milli-Q water or equivalent
Dilute acid: e.g., 0.1% trifluoroacetic acid (TFA) or 1% acetic acid or formic acid
1 mg/ml BNPS-skatole (Pierce; store desiccated at –20°C) in glacial acetic acid (aldehyde-free; Mallinckrodt Specialty Chemicals), prepared immediately before use

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Aluminum foil or opaque container
Beckman Microfuge 11 equipped with type 13.2 fixed-angle rotor, or equivalent microcentrifuge

Additional reagents and equipment for reversed-phase (*UNIT 11.6*) or size-exclusion chromatography (*UNIT 8.3*), electrophoresis (*UNIT 10.1*), or electroblotting to PVDF membranes (*UNIT 10.7*)

1. In a 0.5-ml microcentrifuge tube, solubilize sample containing 1 to 50 µg total protein in solvent such as Milli-Q water or dilute acid (0.1% TFA or 1% acetic or formic acid) to a final protein concentration of 1 mg/ml.

   Which solvent should be used for a particular protein may need to be determined empirically. Additional chaotropic agents—e.g., 8 M urea or 6 M guanidine hydrochloride—may be added to aid solubilization.

2. Add 60 µl BNPS-skatole to 20 µl protein. Cover tube with aluminum foil or place an opaque container over sample tube. Incubate 30 min at room temperature.

   It may be beneficial to increase the incubation time and temperature as well as adding chaotropic agents to the original protein solution to increase the yield from cleavage. If sufficient protein is available, an experiment covering an incubation time of 30 min to 24 hr, at room temperature and 47°C, can be used to optimize the reaction for a specific protein.

   IMPORTANT NOTE: *This reaction must be performed in complete darkness to minimize unwanted side reactions with other reactive amino acid side-chains.*

3. Add 80 µl Milli-Q water to sample to precipitate BNPS-skatole. Centrifuge sample 5 min at 10,000 × *g* (12,000 rpm in Beckman Microfuge 11 with type 13.2 fixed-angle rotor), room temperature.

4. Transfer supernatant to clean 0.5-ml microcentrifuge tube and discard pellet. Lyophilize or dry sample completely in a Speedvac evaporator.

   The resultant pellet may be slightly yellow due to residual reagent and byproducts.

5. Resolubilize sample in an appropriate buffer for separation by reversed-phase (*UNIT 11.6*) or size-exclusion chromatography (*UNIT 8.3*) or for analysis by electrophoresis (*UNIT 10.1*) followed by electroblotting to PVDF membranes (*UNIT 10.7*).

   Some cleavage products can be difficult to resolubilize unless chaotropic agents (e.g., 6 M guanidine hydrochloride or 8 M urea) or detergents (e.g., 1% SDS or 1% cetyltrimethylammonium bromide) are used (see UNIT 1.3 & UNIT 6.1). Sonication is helpful for resolubilization, as is mild heat (i.e., in a 37°C water bath). Use of such agents may necessarily dictate the method chosen for fractionation. For example, detergent-treated samples are best analyzed by electrophoresis, whereas column chromatography should be used for guanidine- or urea-treated samples. Tris-tricine gels are recommended for electrophoretic separation of peptide fragments <10 kDa (UNIT 10.1).

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BASIC PROTOCOL 3

CLEAVAGE AT ASP-PRO PEPTIDE BONDS WITH FORMIC ACID

Formic acid cleaves proteins on the C-terminal side of the Asp residue in aspartic acid–proline (Asp-Pro) peptide bonds. For proteins with multiple Asp-Pro bonds, fragments generated from this reaction can be incomplete (i.e., contain an internal Asp-Pro bond). After digestion with formic acid, resulting peptide fragments are fractionated by HPLC or electrophoresis prior to further characterization.

Materials

1 to 50 µg lyophilized protein sample in capped 0.5-ml microcentrifuge tube
70% formic acid prepared immediately before use from 88% formic acid (Fluka)
Milli-Q water or equivalent

Additional reagents and equipment for reversed-phase (*UNIT 11.6*) or size-exclusion (*UNIT 8.3*) chromatography, electrophoresis (*UNIT 10.1*), or electroblotting to PVDF membranes (*UNIT 10.7*)

1. Add 50 µl of 70% formic acid to lyophilized protein sample. Vortex thoroughly. Incubate 48 hr at 37°C in a chemical fume hood.
2. Add 150 µl Milli-Q water to sample. Lyophilize or dry sample completely.
3. Resolubilize sample in appropriate buffer for separation by reversed-phase (*UNIT 11.6*) or size-exclusion chromatography (*UNIT 8.3*) or for analysis by electrophoresis (*UNIT 10.1*) followed by electroblotting to PVDF membranes (*UNIT 10.7*).

Some cleavage products can be difficult to resolubilize unless chaotropic agents (e.g., 6 M guanidine hydrochloride or 8 M urea) or detergents (e.g., 1% SDS or 1% cetyltrimethylammonium bromide) are used. Sonication is helpful for resolubilization, as is mild heat (i.e., in a 37°C water bath; see UNIT 1.3 & UNIT 6.1). Use of such agents may necessarily dictate the method chosen for fractionation. For example, detergent-treated samples are best analyzed by electrophoresis, whereas column chromatography should be used for guanidine- or urea-treated samples. Tris-tricine gels are recommended for electrophoretic separation of peptide fragments <10 kDa (UNIT 10.1).

BASIC PROTOCOL 4

CLEAVAGE AT ASN-GLY PEPTIDE BONDS WITH HYDROXYLAMINE

Hydroxylamine cleaves proteins on the C-terminal side of Asn residues in asparagine-glycine (Asn-Gly) peptide bonds. For proteins with multiple Asn-Gly peptide bonds, fragments generated from this reaction can be incomplete (i.e., contain an internal Asn-Gly). After digestion with hydroxylamine, resulting peptide fragments are fractionated by HPLC or electrophoresis prior to further characterization.

Materials

1 to 50 µg lyophilized protein sample in a 0.5-ml capped microcentrifuge tube
1.8 M hydroxylamine solution (see recipe)
10% TFA
45°C oven or water bath

Additional reagents and equipment for reversed-phase (*UNIT 11.6*) or size-exclusion chromatography (*UNIT 8.3*)

1. Add 200 µl of 1.8 M hydroxylamine solution to lyophilized protein sample. Vortex thoroughly. Incubate sample 3 hr at 45°C in a chemical fume hood.
2. Allow sample to cool to room temperature and slowly add 10 µl of 10% TFA to quench the reaction.

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3. Fractionate or desalt the sample by reversed-phase (*UNIT 11.6*) or size-exclusion chromatography (*UNIT 8.3*).

The high concentration of salt (1.8 M hydroxylamine) in the reaction mixture makes direct sample analysis by SDS-PAGE extremely problematic. The reaction products can be analyzed by electrophoresis, however, if the sample is desalted prior to analysis (UNIT 8.3). Tris-tricine gels are recommended for electrophoretically separating peptide fragments <10 kDa (UNIT 10.1).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

CNBr, 5 mg/ml in formic acid

CAUTION: *CNBr is extremely toxic. Therefore, all of the following manipulations should be performed as described with extreme caution. Do not transfer dried or solubilized CNBr outside of a chemical fume hood unless it is in a sealed container. Once CNBr has been transferred to a closed microcentrifuge tube, it should be removed from the hood only to be weighed. All other manipulations should be performed inside the hood.*

Immediately before solution is to be used, prepare 1 ml of 70% formic acid by mixing 795 µl of 88% formic acid (Fluka) and 205 µl Milli-Q water in a microcentrifuge tube. Tare analytical balance with a second clean microcentrifuge tube. Transfer one or two crystals of CNBr (Fluka) to tared tube using a clean, dry spatula. Carefully cap microcentrifuge tube and CNBr stock reagent bottle. Weigh CNBr sample and add 70% formic acid to yield a final concentration of 5 mg/ml. Cap tube and vortex carefully until CNBr crystals are solubilized.

CAUTION: *Crystallized CNBr may sublimate, especially after numerous cycles of freezing and thawing or after a long period of storage. It is also hygroscopic. Therefore, it is always advisable to check before use that CNBr is still crystalline. Purchase CNBr in small quantities and store desiccated at –20°C or below.*

Hydroxylamine solution, 1.8 M

Place 5 g hydroxylamine (Fluka) in a 50-ml graduated cylinder. Add 20 ml of 5 M NaOH (pH should be ~7.2). If salt does not dissolve, continue adding NaOH until pH is 7 to 8. Add 1 g Na_2CO_3 and adjust pH to 9 with 12 M HCl. Add Milli-Q water to 40 ml and readjust pH to 9 if needed. Prepare immediately before use.

COMMENTARY

Background Information

The most specific and effective way to fragment proteins into relatively large peptides is by chemical cleavage. CNBr and BNPS-skatole cleave specifically at methionine (Met; Gross, 1967) and tryptophan (Trp; Fontana, 1972) residues, respectively. These two amino acids are in relatively low abundance (Crawford, 1990) and are each encoded by a single codon in the genetic code. As a consequence, protein fragmentation at these residues is desirable because relatively long peptides are obtained (Jay, 1984; Jue and Doolittle, 1985), and is ideally suited for the design of low-degeneracy nucleotide probes.

In addition to the protocols presented in this chapter, chemical cleavage procedures specific for other low-frequency residues (Crawford, 1990) have been successful (Smith, 1994). Proteins can be cleaved at tyrosine (Tyr) residues with *N*-bromosuccinimide (NBS), but this reagent will also cleave at Trp residues. The large excess of NBS required to obtain a reasonable yield typically results in modification of sulfur-containing amino acids [Met and cysteine (Cys)] and histidine (His; Fontana and Gross, 1986). Trp residues can also be cleaved with *o*-iodosobenzoic acid but with a reported specificity and yield less than those of the preferred BNPS-skatole method.

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Proteins can be cleaved at Cys residues by cyanylation with 2-nitro-5-thiocyanobenzoic acid (NTCB) followed by exposure to a borate buffer (pH 9). This residue-specific method produces a good yield of cleavage fragments (Jacobsen et al., 1973; Degani and Patchornik, 1974; Fontana and Gross, 1986). The disulfide linkages (cystine bonds) must first be reduced to free sulfhydryl groups (Cys residues) before proceeding with the NTCB reaction. Unfortunately, the N-termini of the generated fragments will become iminothiazolidinyl residues, resulting in "blocked" peptides that are refractory to sequence analysis by Edman degradation. One report demonstrated the removal of the blocking group with Raney nickel for subsequent N-terminal sequence analysis; however, the amount of protein used was substantial and the resultant amount of free N-terminal product was quite low (Otieno, 1978).

Critical Parameters

A general disadvantage of any chemical cleavage procedure is that chemical fragmentation tends to be incomplete (Smith, 1994), leaving some peptide fragments with undigested internal target residues. One can attempt to increase fragment yield by a combination of increasing chemical reagent concentration, reaction time, and temperature, and adding denaturants during the cleavage reaction. Further, the high concentration of reagents used may cause unwanted modification of non-target amino acid residues in the protein (a problem that is exacerbated if the reagent concentrations are increased as just suggested). Side reactions (e.g., whereby BNPS-skatole may oxidize Cys or Met) can be minimized by performing the cleavage in complete darkness, as described in the protocols. In addition, all reagents must be pure and fresh, and solutions should be stored as recommended and made fresh immediately prior to their use. Taking these few precautions will result in optimal cleavage with minimal side reactions. If possible, mass spectrometry (*UNIT 16.1*) should be used to characterize the resulting cleavage products and will greatly aid in assessing the extent of side reactions. This approach was successfully employed to determine side-reaction modifications formed during solution-phase BNPS-skatole cleavage of proteins and peptides (Vestling et al., 1995).

Successful cleavage and recovery of fragments for sequence analysis can be performed with as little as 5 μg of pure protein. However, obtaining unambiguous protein sequence data requires that the protein sample be pure.

Correct estimation of protein quantity is the single most important factor in determining whether sequence data can be obtained. All too often, an estimated 10 pmol turns out to be 1 pmol. Therefore, care should be taken to prepare adequate amounts of protein for cleavage and to estimate protein concentration accurately. Obtaining an accurate estimate of the amount of protein available can be difficult. The best method involves amino acid compositional analysis (*UNIT 3.2*). This also provides information about the amino acid composition of the protein, which is also important because it should ultimately correspond to the predicted sequence. If the protein concentration cannot be determined by amino acid compositional analysis, estimate the concentration by comparing the sample with known quantities of a standard protein by SDS-PAGE (*UNIT 10.1*). It is best to use a standard protein that approximates the molecular weight and possible secondary structure of the unknown protein. After staining with either Coomassie blue or amido black (*UNIT 10.5*), the gel can be scanned using a densitometer and a linear standard curve can be generated based on the amount of protein versus staining intensity. Analytical SDS-PAGE can also be used to estimate the quantity of an unknown protein prior to committing the remainder of the protein preparation to a particular cleavage method. Molar concentrations can be calculated from quantitation and molecular weight.

Troubleshooting

Commercial proteins of known sequence are useful for monitoring and optimizing the efficiency of chemical cleavage reactions. Individual heavy and light chains of human immunoglobulin G (IgG) proteins can be used to monitor CNBr cleavage; BSA can be used to monitor BNPS-skatole cleavage; bovine brain calmodulin (CAL) can be used to monitor hydroxylamine cleavage at Asn-Gly peptide bonds; and human serum transferrin (HST) can be used to monitor formic acid cleavage at Asp-Pro peptide bonds. Appropriate protein standards must be included as positive controls for any chemical cleavage experiment on any protein; reaction products thus generated are easily analyzed by SDS-PAGE (*UNIT 10.1*). Table 11.4.1 provides the locations of the chemical cleavage sites for the four standard proteins discussed above.

The efficiency of chemical cleavage can be compromised if the protein to be analyzed contains any disulfide bonds. If the sample has

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Table 11.4.1 Commercially Available Proteins Useful as Positive Controls for Chemical Cleavage Reactions[a,b]

Protein	Cleavage reaction/target residue(s)	Location[c]
Human immunoglobulin heavy and light chains	CNBr/Met	Light chain: Met at 4 and 89 (214 residues total)
		Heavy chain: Met at 82, 265, and 459 (478 residues total)
Bovine serum albumin	BNPS-skatole/Trp	Trp at 134 and 212 (582 residues total)
Human serum transferrin	Formic acid/Asp-Pro	Asp-Pro at 90-91 and 548-549 (679 residues total)
Bovine brain calmodulin	Hydroxylamine/Asn-Gly	Asn-Gly at 61-62 and 97-98 (148 residues total)

[a]SDS-PAGE is by far the preferred analytical technique to monitor the reaction. In addition, the reaction products can be electroblotted to a PVDF membrane (*UNIT 10.7*) for N-terminal sequence analysis to assess the efficiency of the chemical cleavage reaction.

[b]Abbreviations: Asn, asparagine; Asp, aspartic acid; BNPS-skatole, 2-(2′-nitrophenylsulfonyl)-3-methyl-3-bromo indolenine; CNBr, cyanogen bromide; Gly, glycine; Met, methionine; Pro, proline; Trp, tryptophan.

[c]The extent of cleavage at each site is not the same, resulting in a skewed distribution of fragments. For a protein with n cleavage sites (n amino acids not at the C-terminus), there are a total of $[(n+1)(n+2)]/2$ possible fragments, $(n+1)$ of which are complete digest fragments, and $[n(n+1)]/2$ of which are incomplete digestion fragments, including the intact, undigested protein substrate (Crimmins et al., 1990).

Cys residues that are involved in disulfide bond formation, it should be reduced and alkylated prior to cleavage to improve cleavage efficiency and yield. This step will also permit unalkylated Cys residues that would be missed by Edman degradation to be identified by sequence analysis. The protein should be denatured by dissolution in 6 M guanidine·HCl or 8 M urea (see *UNIT 6.3*). Reduction is performed under an inert atmosphere (nitrogen or argon) with dithiothreitol (DTT), 2-mercaptoethanol, or tributyl phosphine (the latter is commonly used because of its volatility). It is critical to take extreme care in following all precautions recommended by the manufacturer when using this reagent. Alkylating reagents include iodoacetamide, iodoacetate, and vinyl pyridine, which modify Cys to carboxamidomethyl-Cys, carboxymethyl-Cys, and pyridylethyl-Cys, respectively.

Reduction and alkylation are best performed in solution prior to cleavage: the sample should then be desalted by conventional chromatographic methods (*UNIT 8.3*). An alternative is to use PVDF sample cleanup filters manufactured by Applied Biosystems (ProSpin cartridge) or Millipore. These are easy to use and require no more complex equipment than a centrifuge that can hold a 1.5-ml microcentrifuge tube. The sample mixture is applied, then centrifuged for several hours (depending on the volume and viscosity), and the end result is the protein bound to a PVDF membrane. At this point, the protein sample can be washed and subjected to chemical cleavage for membrane-bound protein (*UNIT 11.5*).

If there is poor or no cleavage with CNBr or BNPS-skatole, the Met or Trp residues may be oxidized. Methionine sulfoxide oxidation can be reversed by the use of *N*-methylmercaptoacetamide (Houghton and Li, 1979), but this method will not reverse methionine sulfone oxidation. Unfortunately, there is no method available for reversing Trp oxidation. Most of these oxidations occur during the isolation and characterization of the protein of interest, and steps should be taken to minimize these occurrences.

Deamidation of Asn residues occurs spontaneously during protein degradation, causing the formation of a succinamide intermediate. If this occurs, hydroxylamine will not cleave at the Asn-Gly peptide bond under the conditions detailed in Basic Protocol 4. However, cleavage will occur if denaturants are included in the cleavage solution (Kwong and Harris, 1994). This ability to differentiate modified Asn residues may be helpful in the elucidation of the primary structure of a protein containing these residues.

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Anticipated Results

The protocols detailed in this unit have been successfully applied to a variety of proteins. As previously discussed, fewer fragments will be generated by chemical cleavage and these fragments will be larger in size than those produced by standard enzymatic treatment. Consequently, in addition to reversed-phase (*UNIT 11.6*), ion-exchange (*UNIT 8.2*), and size-exclusion (*UNIT 8.3*) chromatographies, analytical high-resolution electrophoresis (*UNIT 10.1*) can be used to purify the peptide fragments.

Electrophoresis is often the method of choice for separating small quantities of peptides because it is a technique most laboratories are equipped to perform and the recovery of the cleaved peptides is relatively high. Tris-tricine gels are recommended for peptides of lower molecular weight (<10 kDa) because this buffer system offers good resolution (*UNIT 10.1*).

Electroblotting to PVDF membranes is the only additional step required for preparing the peptides for sequence analysis (*UNIT 10.8*). As a rule of thumb, 100 pmol of pure protein should yield 25 pmol of cleaved peptide on PVDF after electrophoresis and electroblotting (assuming a 50% efficiency for both cleavage and electrotransfer). These numbers may vary, but it is best to start with at least 100 pmol of pure protein.

Time Considerations

In general, allow a full two days for completion of both the cleavage and isolation of peptides. It is best to proceed directly from the cleavage reaction to peptide isolation without stopping in order to minimize protein damage.

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Excellent source of chemical cleavage procedures.

Smith, B. 1994. See above.

Valuable, up-to-date summary of several chemical cleavage procedures.

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Chemical Cleavage of Proteins on Membranes

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer to polyvinylidene difluoride (PVDF) membranes (*UNIT 10.8*) has become the most widely-used method of preparing protein samples for primary structural analysis. Very often SDS-PAGE (*UNIT 10.1*) or two-dimensional isoelectrophoresis/SDS-PAGE (*UNIT 10.4*) is used as a final preparative step for proteins that can only be recovered in very small quantities. The protocols in this unit describe in situ chemical cleavage methods that have been optimized for proteins that are noncovalently bound to PVDF membranes. These procedures can also be used to analyze PVDF-bound protein samples that have already been subjected to Edman degradation. This allows a sample with a blocked N-terminus to be chemically cleaved and resubmitted for sequence analysis to obtain internal sequence data.

Alternatively, the reaction fragments can be eluted from the membrane and fractionated by HPLC or analytical high-resolution electrophoresis (*UNIT 10.1*) prior to further analysis. The fragments generated from chemical digestion tend, on average, to be fewer in number and larger in size than those produced via proteolysis. These chemical cleavage fragments are thus useful as substrate species for secondary cleavage reactions or for directly obtaining internal protein sequence data for use in the subsequent design of DNA probes for genomic or cDNA-library screening.

In this unit, four basic protocols present widely used reactions that are specific and efficient for the in situ chemical cleavage of proteins bound to membranes (related protocols for cleaving proteins in solution, utilizing the same chemical reactions, are presented in *UNIT 11.4*). Cyanogen bromide (CNBr; Basic Protocol 1) cleaves proteins at methionine (Met) residues; 2-(2′-nitrophenylsulfonyl)-3-methyl-3-bromoindolenine (BNPS-skatole; Basic Protocol 2) cleaves proteins at tryptophan (Trp) residues; formic acid (Basic Protocol 3) cleaves proteins at aspartic acid–proline (Asp-Pro) peptide bonds; and hydroxylamine (Basic Protocol 4) cleaves proteins at asparagine-glycine (Asn-Gly) peptide bonds. In general, reactions are chosen based on a priori knowledge of a protein's target residues. If no information is available, perform the reactions in the order listed. In addition, an Alternate Protocol describes CNBr cleavage of PVDF-bound protein previously analyzed by Edman degradation. Finally, a Support Protocol discusses preferred methods of separating and analyzing peptide fragments generated by the chemical cleavage reactions described in the basic protocols.

CAUTION: Most chemicals used in the following protocols are toxic. Therefore, it is imperative that good laboratory practice be followed. All procedures and incubations should be performed in a well-ventilated chemical fume hood and laboratory personnel should wear appropriate protective clothing, including safety glasses and gloves. All chemical waste should be properly disposed of according to local regulations.

CLEAVAGE AT THE C-TERMINUS OF MET RESIDUES WITH CNBr

BASIC PROTOCOL 1

CNBr cleaves proteins on the C-terminal side of unoxidized methionine (Met) residues. For proteins with multiple Met residues, fragments generated from this reaction can be incomplete (i.e., contain an internal unmodified Met or homoserine). After the reaction, the PVDF-bound peptides may be sequenced directly (if multiple N-termini are expected from a protein that contains more than one Met residue) or eluted from the PVDF membrane (*UNIT 10.7*) and fractionated prior to further characterization. Additionally, if the N-terminus of a protein is thought to be blocked (because no sequence could be obtained

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from the first sequencing attempt although a sufficient amount of the protein was analyzed), in situ CNBr cleavage can be used to confirm N-terminal blockage by directly sequencing the PVDF membrane after the cleavage reaction.

Materials

Dried PVDF membrane containing electroblotted protein sample (*UNIT 10.7*)
160 mg/ml CNBr in 70% formic acid (prepared as in *UNIT 11.4*, but with different concentration)
Milli-Q water or equivalent

Clean glass plate
Aluminum foil or opaque container
47°C water bath or oven (optional)

Additional reagents and equipment for analysis and separation of cleavage fragments (see Support Protocol)

1. Feather PVDF membrane containing electroblotted protein by making parallel cuts running most of its length with a clean razor blade to give a pattern similar to that of a rake head.

   Feathering the PVDF membrane increases its surface area.

2. Place feathered membrane in a clean 0.5-ml microcentrifuge tube and add 150 µl CNBr/formic acid. Cap tube, then wrap completely with aluminum foil or place in an opaque container. Incubate with agitation in a chemical fume hood either 24 to 48 hr at room temperature or 1 hr at 47°C.

   Traditionally, CNBr incubations are performed for 24 to 48 hr at room temperature; however, we find that the shorter incubation at 47°C produces comparable results for most proteins.

   IMPORTANT NOTE: *This reaction must be performed in complete darkness to minimize unwanted side reactions with other reactive amino acid side-chains.*

3. Neutralize unused CNBr by slowly transferring remaining CNBr to a 5-ml tube containing 5 to 10 vol of 10 M NaOH. Rinse the pipet with 10 M NaOH before discarding.

   CAUTION: *Reaction of CNBr with NaOH is exothermic, generating heat and bumping of the solution. Therefore, add the CNBr solution slowly to minimize violent reactions. Once the CNBr has been neutralized, discard it according to halogen chemical waste disposal procedures.*

4. Dry entire reaction mixture (membrane plus liquid) 1 to 2 hr in a Speedvac evaporator.

5. Add 50 µl Milli-Q water to dried sample. Vortex and dry sample again.

   The water wash removes residual acid; this is important for subsequent electrophoresis or chromatography.

6. Analyze peptide sequence directly from membrane or separate peptide fragments (see Support Protocol).

   CAUTION: *After lyophilization or drying, defrost the cold trap and neutralize its contents by adding 10 M NaOH until the pH reaches ≥7.0. Discard contents according to halogen chemical waste disposal procedures.*

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CNBr CLEAVAGE OF PVDF-BOUND PROTEIN PREVIOUSLY ANALYZED BY EDMAN DEGRADATION

ALTERNATE PROTOCOL

This protocol is used to obtain internal protein sequence from a PVDF-bound protein that has been previously sequenced via Edman degradation. It combines in situ CNBr cleavage and *o*-phthalaldehyde (OPA) blockage to obtain protein sequence data from samples that contain a small amount of protein. It should be noted that this cleavage is relatively inefficient, usually generating a small number of N-terminal (1 to 4) that can be sequenced. In favorable circumstances, one gets only a single or major sequence, thus allowing unambiguous assignment.

Additional Materials (also see Basic Protocol 1)

25% (w/v) CNBr in 70% formic acid (prepared as in *UNIT 11.4*, but with different concentration)
Preconditioned polybrene-treated glass-fiber filters

1. Feather PVDF membrane containing bound protein sample using a clean razor blade (see Basic Protocol 1, step 1). Place membrane on top of preconditioned polybrene-treated glass-fiber filter.

   Preconditioning of polybrene-treated glass-fiber filters is done as a prerequisite to protein sequencing. Refer to Atherton et al. (1993a) for details regarding sequence analysis of proteins.

2. Apply 30 to 42 µl of 25% CNBr/formic acid to sample until filter and PVDF membrane are saturated.

3. Wrap wet filters in Parafilm to prevent evaporation, then wrap with aluminum foil or cover with opaque container. Incubate 24 hr at room temperature in chemical fume hood.

   The glass reaction-cartridge block from an ABI sequencer can be used as the support for this reaction. Alternately, the filter and PVDF membrane can be placed on top of a piece of Parafilm large enough to create an envelope around the membrane and filter when folded. Take care to avoid contact between the Parafilm and the membrane.

   IMPORTANT NOTE: *This reaction must be performed in complete darkness to minimize unwanted side reactions with other reactive amino acid side-chains.*

4. Using a clean razor blade, slice a hole in Parafilm and place the packet in a Speedvac evaporator. Dry sample for 10 min.

5. Prepare PVDF membrane for sequence analysis using a fresh preconditioned polybrene-treated glass-fiber filter.

6. Subject ~25% of membrane to Edman degradation to ascertain the positions of Pro residues.

7. Block other residues on remaining membrane with OPA. Subject blocked membrane to further sequence analysis via Edman degradation.

   OPA blockage will result in a sequence beginning with one or more Pro residues. Consult Brauer et al. (1984) for specific details regarding modification of sequencer cycles to optimize OPA blockage, and Atherton et al. (1993a) for details regarding sequence analysis.

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CLEAVAGE AT THE C-TERMINUS OF TRP RESIDUES WITH BNPS-SKATOLE

BNPS-skatole cleaves proteins on the C-terminal side of unoxidized tryptophan (Trp) residues. For proteins with multiple Trp residues, fragments generated from this reaction can be incomplete (i.e., contain an internal unmodified or oxidized Trp). After the reaction, the PVDF membrane may be sequenced directly (if multiple N-termini are expected because the protein contains more than one Trp) or the fragments eluted from the PVDF membrane (*UNIT 10.7*) and fractionated prior to further characterization. Additionally, if the N-terminus of a protein is thought to be blocked (because no sequence is obtained from the first sequencing attempt although a sufficient amount of protein was analyzed), in situ BNPS-skatole cleavage can be used to confirm N-terminal blockage by directly sequencing the PVDF-bound fragments after the cleavage reaction.

Materials

Dried PVDF membrane containing electroblotted protein sample (*UNIT 10.7*)
Milli-Q water or equivalent
1 mg/ml BNPS-skatole (Pierce; store desiccated at –20°C) in 75% glacial acetic acid (aldehyde-free, Mallinckrodt Specialty Chemicals), prepared immediately before use

Clean glass plate
Aluminum foil or opaque container
47°C water bath or oven
Beckman Microcentrifuge equipped with type 13.2 fixed-angle rotor, or equivalent microcentrifuge

Additional reagents and equipment for analysis and separation of cleavage fragments (see Support Protocol)

1. Feather PVDF membrane containing protein of interest using a clean razor blade (see Basic Protocol 1, step 1).
2. Place feathered membrane in clean 1.5-ml microcentrifuge tube and add 100 µl BNPS-skatole. Cap tube, then wrap with aluminum foil or place in an opaque container. Incubate 1 hr at 47°C with occasional agitation.

   IMPORTANT NOTE: *This reaction must be performed in complete darkness to minimize unwanted side reactions with other reactive amino acid side-chains.*
3. Remove liquid portion of sample to a clean 0.5-ml microcentrifuge tube and save until use in step 6.
4. Add 500 µl Milli-Q water to tube containing PVDF membrane and vortex vigorously. Aspirate water from membrane and discard.
5. Repeat wash four more times. Dry membrane in a Speedvac evaporator.
6. Add 100 µl Milli-Q water to liquid sample from step 3. Centrifuge 5 min at 10,000 × *g* (12,000 rpm in Beckman Microfuge with type 13.2 fixed-angle rotor), room temperature.

   This step precipitates BNPS-skatole from the reaction mixture.
7. Transfer supernatant to clean microcentrifuge tube and dry in a Speedvac evaporator.
8. Add 100 µl Milli-Q water to dried sample and vortex ~5 min. Dry resulting solution in a Speedvac evaporator.

   Initial pellet may appear yellow due to residual reagent and byproducts.

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9. Analyze sample (using both dried membrane and liquid sample) by direct sequence analysis *or* separate the peptide fragments (see Support Protocol).

CLEAVAGE AT ASP-PRO PEPTIDE BONDS WITH FORMIC ACID

BASIC PROTOCOL 3

Formic acid cleaves proteins primarily at the C-terminal side of the Asp residue in aspartic acid–proline (Asp-Pro) peptide bonds. However, secondary cleavages may occur at acid-labile bonds such as aspartic acid–threonine (Asp-Thr) and aspartic acid–serine (Asp-Ser). In addition, for proteins with multiple Asp-Pro peptide bonds, fragments generated from this reaction can be incomplete (i.e., contain internal Asp-Pro peptide bonds). Therefore, sequence analysis following dilute acid cleavage is usually accompanied by treatment with *O*-phthalaldehyde (OPA) to block all N-termini that may have been generated except for the N-terminal Pro. Alternately, if sample quantities allow, the fragments can be eluted from the PVDF membrane (*UNIT 10.7*) and fractionated prior to further characterization.

Materials

Dried PVDF membrane containing electroblotted protein sample (*UNIT 10.7*)
88% formic acid (Fluka)

Preconditioned polybrene-treated glass-fiber filter
45°C oven or water bath

Additional reagents and equipment for analysis and separation of cleavage fragments (see Support Protocol)

1. Feather PVDF membrane containing protein sample using a clean razor blade (see Basic Protocol 1, step 1).
2. Place feathered membrane and corresponding preconditioned polybrene-treated glass-fiber filter on a sheet of Parafilm large enough to form an envelope around membrane and filter.
3. Apply 88% formic acid to sample until membrane and filter are saturated.
4. Cover sample completely with Parafilm and seal to prevent evaporation.
5. Incubate sealed packet 24 hr at 45°C in a chemical fume hood.
6. Remove sample from oven, place in chemical fume hood, and carefully cut Parafilm to expose membrane and filter to air.
7. Dry sample 5 min in a Speedvac evaporator.
8. Analyze sample using direct sequence analysis *or* separate the peptide fragments (see Support Protocol).

CLEAVAGE AT ASN-GLY PEPTIDE BONDS WITH HYDROXYLAMINE

BASIC PROTOCOL 4

Hydroxylamine cleaves proteins on the C-terminal side of Asn residues in asparagine-glycine (Asn-Gly) peptide bonds. For proteins with multiple Asn-Gly bonds, fragments generated from this reaction can be incomplete (i.e., contain an internal Asn-Gly peptide bond). After the reaction, the PVDF membrane may be sequenced directly (if multiple N-termini are expected because the protein contains more than one Asn-Gly peptide bond) or the fragments eluted from the PVDF membrane (*UNIT 10.7*) and fractionated prior to further characterization. Additionally, if the N-terminus of a protein is thought to be blocked (because no sequence was obtained from the first sequencing attempt although a sufficient amount of protein was analyzed), in situ hydroxylamine cleavage can be used

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to confirm N-terminal blockage by directly sequencing the PVDF membrane after the cleavage reaction.

Materials

Dried PVDF membrane containing electroblotted protein sample (*UNIT 10.7*)
2 M hydroxylamine solution (see recipe)
Milli-Q water or equivalent
45°C oven or water bath

Additional reagents and equipment for analysis and separation of cleavage fragments (see Support Protocol)

1. Feather PVDF membrane containing protein sample using a clean razor blade (see Basic Protocol 1, step 1).
2. Place feathered PVDF membrane in clean 1.5-ml microcentrifuge tube. Working in a chemical fume hood, add 20 to 30 µl hydroxylamine solution until membrane and filter are wet.
3. Cap tube and incubate 9 hr at 45°C in a chemical fume hood with agitation ad libitum.
4. Add 1 ml Milli-Q water to sample. Vortex vigorously. Aspirate water from membrane and discard.
5. Repeat wash two more times. Dry membrane in Speedvac evaporator or air dry.
6. Analyze sample using direct sequence analysis *or* separate the peptide fragments (see Support Protocol).

SUPPORT PROTOCOL

ANALYSIS AND SEPARATION OF CLEAVAGE FRAGMENTS

After performing any of the in situ cleavage reactions described in the four basic protocols, the resultant fragments can be analyzed by several different methods. The fragments can be separated by SDS-PAGE (*UNIT 10.1*) or two-dimensional electrophoresis (*UNIT 10.4*). They can then be eluted from the gel for direct sequence analysis, or electroblotted to PVDF or another suitable membrane (*UNIT 10.7*) for sequence analysis on the membrane. Cleaved fragments from any of the chemical cleavage protocols can also be purified by reversed-phase (*UNIT 11.6*) or size-exclusion chromatography (*UNIT 8.3*) following elution from the PVDF membrane (*UNIT 10.7*; Szewczyk and Summers, 1988).

Direct Sequence Analysis

If no additional purification of the cleavage fragments is necessary, subject the membrane to N-terminal sequence analysis, with the proviso, of course, that multiple N-termini may be observed. This can be useful, however, in confirming the presence of a blocked N-terminus and may yield interpretable sequence information due to variable cleavage or sequence yields.

NOTE: Regardless of the in situ cleavage method used, an ethyl acetate wash cycle should be used prior to the first sequencing cycle (Atherton et al., 1993a).

For BNPS-skatole-cleaved protein fragments: Combine the dried membrane and the dried original liquid portion of the in situ digest, the latter resuspended in a minimal volume of 0.1% (v/v) TFA/50% (v/v) acetonitrile. Load the mixture onto a sequencer for N-terminal sequence analysis. The results are subject to the same caveats stated above.

For proteins cleaved with formic acid at Asp-Pro bonds: Use OPA to block the N-termini of other sites that may have been generated with the formic acid (Brauer et al., 1984). Load onto a sequencer for N-terminal sequence analysis.

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Elution of Cleaved Fragments by a Second Round of SDS-PAGE

Add 50 µl of 2% SDS/1% Triton X-100/50 mM Tris·HCl (pH 9.1) elution buffer to the PVDF membrane containing cleaved fragments. Incubate 90 min at room temperature with occasional vortexing. Remove membrane from elution buffer and add a minimal volume of a concentrated stock of an appropriate SDS-PAGE loading buffer (Crimmins et al., 1990). Load on an SDS-polyacrylamide gel and perform electrophoresis (*UNIT 10.1*); if peptides <10 kDa are expected, use of Tris-tricine gels is recommended. Elute the cleaved fragments directly from the gel for further digestion or sequence analysis *or* electroblot fragments to PVDF membrane for subsequent N-terminal sequence analysis (*UNIT 10.7*). In addition, for larger cleavage fragments, enzymatic digestion can be performed to recover smaller fragments for N-terminal sequence analysis or mass-spectrometric analysis.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Hydroxylamine solution, 2 M

Weigh 5.5 g hydroxylamine·HCl (Fluka) into a 100-ml beaker. Slowly add 4.5 M LiOH, stirring vigorously, until hydroxylamine salt is completely dissolved. Adjust pH to 11.5 with 4.5 M LiOH. Add Milli-Q water (or equivalent) to 40 ml. Prepare immediately before use.

LiOH is used instead of NaOH because it is volatile and hence more easily removed.

COMMENTARY

Background Information

Protein structural analysis requires the integration of peptide isolation techniques with amino acid sequence determination. A problem that frequently arises when working with small quantities of protein is that the sample can be lost between isolation and sequence determination. Transferring electrophoretically separated proteins to membrane supports through electroblotting has become a standard technique for efficient recovery of proteins from the gel matrix (*UNIT 10.7*; Matsudaira, 1987; LeGendre, 1990).

The protocols detailed in this unit have been optimized for chemical cleavage of proteins bound to PVDF membranes. Alternatively, these protocols can be used for proteins bound to cationic-derived polyvinylidene fluoride membranes (CD-PVDF) or spotted onto glass-fiber filters. Refer to the original literature cited for each protocol for specific details regarding in situ cleavage of proteins on these membranes (Simpson and Nice, 1984; Scott et al., 1988; Miczka and Kula, 1989; Patterson et al., 1992; Landon, 1977; Bauw et al., 1988; Crimmins et al., 1990; Wadsworth et al., 1992; and Kwong and Harris, 1994). Nitrocellulose, often used for protein binding studies, is not stable when exposed to the chemicals used in these protocols and should not be used.

There are several advantages to working directly from PVDF-bound protein. Preparing the protein involves no more than electrophoresis followed by electroblotting to the PVDF membrane. PVDF-bound protein can be subjected to sequence analysis prior to chemical cleavage, which permits N-terminal sequencing. If the N-terminal appears blocked, chemical cleavage can be performed and the protein sequenced directly from the same PVDF membrane. In all likelihood, a peptide sample will contain multiple N-termini, but chemical cleavage will provide confirmation that the protein is present and may provide valuable internal sequence data. As detailed in the alternate CNBr protocol, OPA treatment (Brauer et al., 1984) can provide amino acid sequence data starting from very small sample quantities (Wadsworth et al., 1992).

Two variations of the BNPS-skatole cleavage protocol have been reported. The first (described in Bauw et al., 1988) uses minimally different cleavage conditions (80% acetic acid and a 36-hr incubation at room temperature). However, the original reaction supernatant is not saved and the PVDF is sequenced directly

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after digestion (see Support Protocol). This may result in lower recovery of the cleaved peptides. In the second procedure (Patterson et al., 1992), digested fragments are eluted from a CD-PVDF membrane by incubation for 1 hr at room temperature with 70% formic acid; the eluate is then combined with the initial reaction mixture supernatant prior to further analysis.

Critical Parameters

The protocols described here have been performed on protein samples ranging from 1 to 100 µg, with an average of 10 µg. This quantity of protein is usually obtained from one to ten stained or unstained bands of electroblots from an SDS-PAGE minigel (*UNIT 10.1*) or ten to twenty spots from an electroblot of a two-dimensional gel (*UNIT 10.4*). Although there is no clear consensus as to how important density is to the success of cleavage, keeping the protein as concentrated as reasonable without compromising resolution is recommended.

In general, either "high-retention" or "low-retention" PVDF membranes (Mozdzanowski and Speicher, 1992) can be used for these in situ chemical cleavage protocols, although the "low-retention" type of PVDF membranes yield better peptide recoveries. CD-PVDF has also been used and may have some advantages for the successful elution of larger peptides (Patterson et al., 1992). Proteins bound to PVDF can be stained with amido black, Coomassie blue, or Ponceau S (*UNIT 10.8*) or radiolabeled with the isotope of choice. Proteins bound to CD-PVDF must be negatively stained, as the cationic nature of this membrane prohibits the use of standard protein stains.

All cleavage solutions should be stored as recommended and made fresh immediately prior to use with pure, fresh reagents. A general disadvantage of any chemical cleavage procedure is that chemical fragmentation tends to be incomplete and the high concentration of reagents used may cause unwanted modification of nontarget amino acid residues in the protein. One can attempt to increase fragment yield by a combination of increasing chemical reagent concentration (with the caveat discussed above), reaction time, and temperature, and adding denaturants during the cleavage reaction. These side reactions can be minimized by performing the cleavage in complete darkness, as detailed for both the CNBr and BNPS-skatole protocols. If possible, mass spectrometry (*UNIT 16.1*) should be used to characterize the resulting cleavage products and will greatly aid in assessing the extent of side reactions.

Troubleshooting

Consult the Troubleshooting section of *UNIT 11.4* for guidelines on sample preparation and optimization of the chemical cleavage reactions.

It is important to include an appropriate protein standard as a positive control in any chemical cleavage experiment. See Table 11.4.1 for a list of commercially available protein standards.

If the protein to be cleaved has cysteine (Cys) residues that are involved in disulfide bonds, it is strongly suggested that the sample be reduced and alkylated prior to cleavage (see Troubleshooting, *UNIT 11.4*).

Reduction and alkylation should be performed in solution and the sample desalted using either an Applied Biosystems Prospin PVDF cartridge or a Millipore PVDF sample clean-up cartridge. This allows transferral of protein to PVDF using centrifugation while removing salts and other contaminants that accumulate during sample preparation or modification. Alternately, reduction and alkylation can be performed directly on PVDF (Atherton et al., 1993b) or glass-fiber filters (Andrews and Dixon, 1987). When working with PVDF membranes, it is always advisable to wear talc-free gloves at all times. Please consult *UNIT 10.7* for additional troubleshooting information.

Anticipated Results

Successful cleavage and recovery of fragments for primary structural analysis can be performed with a little as 5 µg of pure protein. However, it is impossible to overemphasize the importance of having a pure protein for obtaining unambiguous protein sequence data.

It should be emphasized that chemical cleavages are often incomplete reactions, and typically generate larger peptide fragments than those obtained with enzymatic digestion. Therefore, they may not be the optimal methods for obtaining a definitive peptide map, because neither cleavage nor recovery is 100%. As a rule of thumb, an initial protein sample of 100 pmol should yield 25 pmol of a resultant peptide on PVDF membranes after electrophoresis and electroblotting (assuming a 50% efficiency for both cleavage and electrotransfer). Of course, recovery can be higher or lower, but it's best to start with at least this much protein.

We have found that for the larger fragments generated using chemical in situ cleavage protocols, the efficiency of elution from PVDF is generally better for the less sensitive and more weakly bound Ponceau-S-stained bands than

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with other methods. Also, if the cleaved fragments will be subjected to an additional purification step, Ponceau-S-stained protein samples are preferred. If the additional round of purification is gel electrophoresis, the type of stain does not matter, and all are equally eluted from the PVDF (Szewczyk and Summers, 1988) into the gel.

Time Considerations

In general, two days is sufficient for the completion of both the cleavage and elution of fragments for further characterization. Once the PVDF-bound protein is cleaved and dried, it can be stored at –20°C for a short period (several days) until elution or sequence analysis is performed. However, excess reagent remaining on the PVDF membrane may cause unwanted modification of the protein. Therefore, the fragments should be eluted or the PVDF mixture analyzed as soon as possible following cleavage.

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Valuable, up-to-date summary of several chemical cleavage procedures.

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Reversed-Phase Isolation of Peptides

Reversed-phase high-performance liquid chromatography (HPLC) is a fundamental tool for the isolation and analysis of peptides. Peptides are separated on a hydrophobic stationary phase and eluted with a gradient of increasing organic solvent concentration. Peptides in 5- to 500-pmol quantities are separated on a narrow-bore (2-mm-i.d.) or microbore (1-mm-i.d.) column (see Basic Protocol 1). Separation of peptides in quantities <5 pmol can be accomplished using capillary HPLC columns (see Basic Protocol 2). Capillary HPLC columns, however, require a gradient flow rate of 3 to 5 µl/min, which most current HPLC pumps cannot attain without modifications. A procedure is therefore provided for constructing a capillary HPLC system using readily available components (see Support Protocol).

HPLC peaks that appear to be symmetrical may actually contain coeluting peptides. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (see Basic Protocol 3 and Alternate Protocol; also see *UNITS 16.1 & 16.2*) and capillary electrophoresis (see Basic Protocol 4; also see *UNIT 10.9*) can be used to analyze a small portion of an HPLC fraction and to determine the number of components present in a small sample. These methods can be utilized to screen fractions prior to automated sequencing.

REVERSED-PHASE PEPTIDE SEPARATION AT THE 5- TO 500-PMOL LEVEL

BASIC PROTOCOL 1

A 2-mm narrow-bore column requires a flow rate of 100 to 200 µl/min. Many commercial HPLC instruments are capable of providing flow rates in this range, although some have tubing with a large internal volume that may add excessive delay time to the separation. Some HPLC instruments that are suitable without modification for 1- and 2-mm columns are listed in the materials section below. Many older HPLC instruments can be utilized for 2-mm columns, but most cannot reproducibly deliver the 50- to 100-µl/min flow rates required by 1-mm columns.

Materials

Mobile-phase solvent A: 0.1% (v/v) trifluoroacetic acid (TFA; Pierce) in Milli-Q water (TFA sold for protein sequencing may not be suitable because some manufacturers add antioxidants that can generate artifacts)

Mobile-phase solvent B: 0.07% to 0.1% (v/v) TFA (Pierce) in acetonitrile or 1- or 2-propanol (Burdick & Jackson or Baker; HPLC-grade)

Solvent modifier: TFA (Pierce)

Milli-Q grade water or equivalent (distilled water is not suitable)

Peptide sample

HPLC peptide standards, commercial (e.g., PE Applied Biosystems) *or* tryptic digest (see Troubleshooting), for testing column

HPLC system (e.g., Hewlett-Packard HP-1090 liquid chromatograph; PE Applied Biosystems model 170A; Michrom BioResources Ultrafast Microprotein Analyzer; Beckman System Gold)

Detector flow cell (see recipe)

2-channel strip-chart recorder (Kipp & Zonen or equivalent)

C18, C8, or C4 reversed-phase columns, 300 Å, 1- or 2-mm i.d. (e.g., SynChrom or Vydac; many other columns from numerous manufacturers can be used)

Contributed by William J. Henzel and John T. Stults

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1. Prepare and degas the mobile phase and set up an appropriate gradient of mobile-phase solvents A and B. Select a gradient based on number of peaks expected and resolution desired.

   Normal gradient: 0% to 70% solvent B in 70 min
   Fast gradient: 0% to 70% solvent B in 35 min
   Slow gradient: 0% to 70% solvent B in 90 to 140 min.

   A normal, fast, or slow gradient should be used when the expected number of peaks is ~30 to 60, <30, or >60, respectively.

2. Program flow rate to 200 µl/min or 100 µl/min for 2-mm- or 1-mm-i.d. columns, respectively. Set detector to 0.1 and 0.02 AUFS and the wavelengths on chart recorder to 214 and 280 nm for channels 1 and 2, respectively. Equilibrate column with initial conditions and establish a flat baseline.

   The 280-nm pen on the chart recorder should be set to a scale five times more sensitive than the 214-nm pen. If only one wavelength is available, use 214 nm.

3. Fill injector loop with starting solvent and run a blank gradient consisting of the same gradient as will be utilized for separation of sample.

   This will wash the column, removing any peptides that may be present from previous injections.

4. Prepare sample for injection as follows (or prepare peptide standards, when using a new column or when troubleshooting):
   a. If the sample contains organic solvent, lower the organic solvent concentration to <10% (v/v) by evaporating it in a Speedvac evaporator or by diluting it with water or aqueous buffer.
   b. Check that the sample volume is appropriate for the volume of the injector sample loop; if not, either reduce the sample volume by evaporation or change to a larger sample loop.
   c. Spin the sample in a microcentrifuge to remove particulates prior to injection.

   The presence of organic solvent in the sample may prevent peptides from adsorbing to the stationary phase.

   The size of the sample loop will not have a significant effect on the separation. However, if a larger loop is used, an appropriate delay should be added to the gradient program to allow the entire sample volume to load on the column and to allow salts that may be in the sample to wash off the column before the gradient begins.

5. Change injector to load position and rinse injector with starting solvent (mobile-phase solvent A).

   If the sample loop was switched from inject to load position during the gradient run, the sample loop will contain organic solvent. If the sample only partially fills the sample loop, the presence of organic solvent in the loop might prevent the sample from binding to the column; rinsing with starting solvent averts this.

6. Inject sample, being careful to avoid injecting air into sample loop.

   Air that is injected onto the column can produce air bubbles that may lodge in the detector.

7. Prepare a rack with 1.5-ml microcentrifuge tubes for collecting peak fractions. Number tubes with felt-tipped pen.

8. Calculate the delay for peak collection by dividing the volume of tubing exiting the flow cell by the flow rate.

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Table 11.6.1 Volumes of Tubing Exiting Flow Cell

Tubing inner diameter	Volume (µl/cm)
0.075-mm (75-µm) FSC	0.045
0.005-in. (127-µm) PEEK	0.127
0.007-in. (178-µm) PEEK	0.249

This is particularly important to prevent miscollection of peaks with low flow rates (<200 µl/min). Table 11.6.1 provides a list of volumes for different diameters of PEEK and FSC tubing.

Powder-free gloves should be worn during peak collection to avoid sample contamination by free amino acids.

9. Collect peaks by monitoring absorbance change on a strip-chart recorder and allowing appropriate delay time (calculated in step 8) before changing tubes.

   A stopwatch can be used to accurately measure the delay time when collecting poorly resolved peaks. Data systems on most HPLC systems have signal processing delays that can make manual peak collection difficult. Monitoring a strip-chart recorder provides a real-time measurement of absorbance and eliminates this problem.

REVERSED-PHASE PEPTIDE SEPARATION AT ≤5 PMOL

BASIC PROTOCOL 2

Separation of peptides that are present in quantities ≤5 pmol requires the use of a capillary column. These columns have internal diameters <1 mm and provide high-resolution peptide mapping of peptides collected from in situ digests of proteins electroblotted from one- and two-dimensional gels. A 0.32-mm-i.d. capillary column is capable of separating peptides at the 500-fmol level, but requires a flow rate of 3 to 5 µl/min. Only one commercial HPLC system currently available can deliver gradient flow rates in this range; other HPLC pumps can be adapted to deliver capillary gradient flow rates using a flow splitter (see Support Protocol).

Materials

Solvent A: 0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water
Solvent B: 0.05% to 0.1% (v/v) TFA in acetonitrile
Peptide sample

Detector flow cell (LC Packings)
Strip-chart recorder (Kipp & Zonen or equivalent)
Capillary HPLC system (see Support Protocol)
Graduated 10-µl Hamilton glass syringe connected to outlet of flow cell with 0.25-mm-i.d. Teflon tubing (LC Packings)
Two stopwatches

Additional reagents and equipment for reversed-phase peptide separation at 5 to 500 pmol (see Basic Protocol 1) and capillary HPLC system assembly (see Support Protocol)

1. Prepare and degas the mobile phase (see Basic Protocol 1, step 1).
2. Set detector to 0.1 AUFS and the wavelength on the chart recorder to 195 nm.
3. Equilibrate capillary column in solvent A until a flat baseline is established. Connect a graduated 10-µl Hamilton glass syringe to the outlet tubing of capillary flow cell with 0.25-mm-i.d. Teflon tubing. Measure the time it takes for liquid to reach a given volume and calculate flow rate as follows: flow rate (in µl/min) = volume/time.

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4. Accurately measure the length of tubing from the flow cell to its outlet end and calculate the delay according to the following formula: delay (min) = volume of outlet tubing (µl)/flow rate (µl/min).

   See Table 11.6.1 for a list of tubing internal volumes. A typical delay time is 35 sec.

5. If necessary, adjust the flow rate to between 3 and 5 µl/min (see Support Protocol, step 1).
6. Run blank gradient, prepare sample, and inject (see Basic Protocol 1, steps 3 to 6).
7. Collect peaks in 0.5-ml microcentrifuge tubes using two stopwatches. Mark the start of the peak with one stopwatch and time the end of the peak with the second watch.

   Peak collection is the most difficult aspect of preparative capillary HPLC and requires a little practice. However, the degree of peak purity will be surprisingly high for closely eluting peaks if the delay time is calculated accurately. Because the flow cell is a length of 75-µm fused silica capillary tubing, only very minor resolution loss occurs as the peaks travel from the end of the column to the outlet of the detector tubing.

   Only one stopwatch will be needed if the capillary flow cell is modified as described for capillary HPLC assembly (see Support Protocol, step 3b).

SUPPORT PROTOCOL

CAPILLARY HPLC SYSTEM ASSEMBLY

Peptide separations at the low picomole level require columns with an i.d. of <1 mm, which are called capillary columns. A 0.32-mm-i.d. capillary column is capable of separating peptides at the 500-fmol level. However, these columns require a flow rate of 3 to 5 µl/min, and at present only one commercial HPLC system can deliver gradient flow rates in this range without modifications. Using the material listed below, it is quite easy to construct or modify an existing HPLC using readily available components. Perkin-Elmer has recently introduced a capillary pump and Waters Associates is developing a capillary HPLC pump that should be available soon.

Materials

Capillary flow cell (LC Packings)
Piston- or syringe-pump splitter (LC Packings) *or* laboratory-constructed splitter: 1/16 in. tee (Valco); fingertight fittings; 0.012-in.-i.d. sleeve for capillary tubing; fingertight fitting for 0.012-in. sleeve (Upchurch Scientific); and 150 cm of 0.05-mm-i.d., 300-mm-o.d. FSC tubing (Polymicro Technologies) *or* 0.250-mm-i.d. Teflon tube inserted into a PEEK fingertight nut and ferrule
Capillary pump (Perkin-Elmer 140-D) *or* HPLC piston pump (see Basic Protocol 1) for use with an LC Packings splitter, *or* HPLC syringe pump (PE Applied Biosystems models 120A, 140A, or 170A) for use with a laboratory-constructed splitter
Injector equipped with a 20-µl loop (Rheodyne)
In-line precolumn filter (Upchurch Scientific)
0.32-mm × 15-cm C18 column (LC Packings, Keystone Scientific, Metachem Technologies, or Micro-Tech Scientific)
Detector flow cell (PE Applied Biosystems, Perkin-Elmer, or Hewlett-Packard)

1a. *If using a capillary pump that can provide a flow rate of 3 to 5 µl/min:* Proceed to step 3a or 3b.

1b. *If assembling a splitter:* Connect a 150-cm length of 0.05-mm-i.d. FSC tubing to Valco tee with a 0.012-in.-i.d. sleeve *or* with a 0.250-mm-i.d. Teflon tube inserted into a PEEK fingertight nut and ferrule. Connect the inlet side of splitter to HPLC pump with 0.005-in.-i.d. PEEK tubing.

   A schematic diagram of the capillary fittings and the splitter assembly is shown in Figure 11.6.1.

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A splitter must be connected to the HPLC pump to allow the flow rate to be reduced from 50 to 200 µl/min to the 3 to 5 µl/min required for a 0.32-mm-i.d. column. A splitter can be purchased commercially or assembled as described. The split ratio is controlled by varying the flow rate from the pump or by adjusting the length of the 0.05-mm-i.d. capillary tubing connected to the Valco tee. The pump flow rate should be set to the lowest flow that the pump can reliably deliver. A homemade splitter requires a pulse-free pump that can reliably deliver a flow rate of <200 µl/min. PE Applied Biosystems syringe pumps can accurately deliver a pulse-free flow rate of 70 µl/min. These pumps have a dynamic mixer and a static

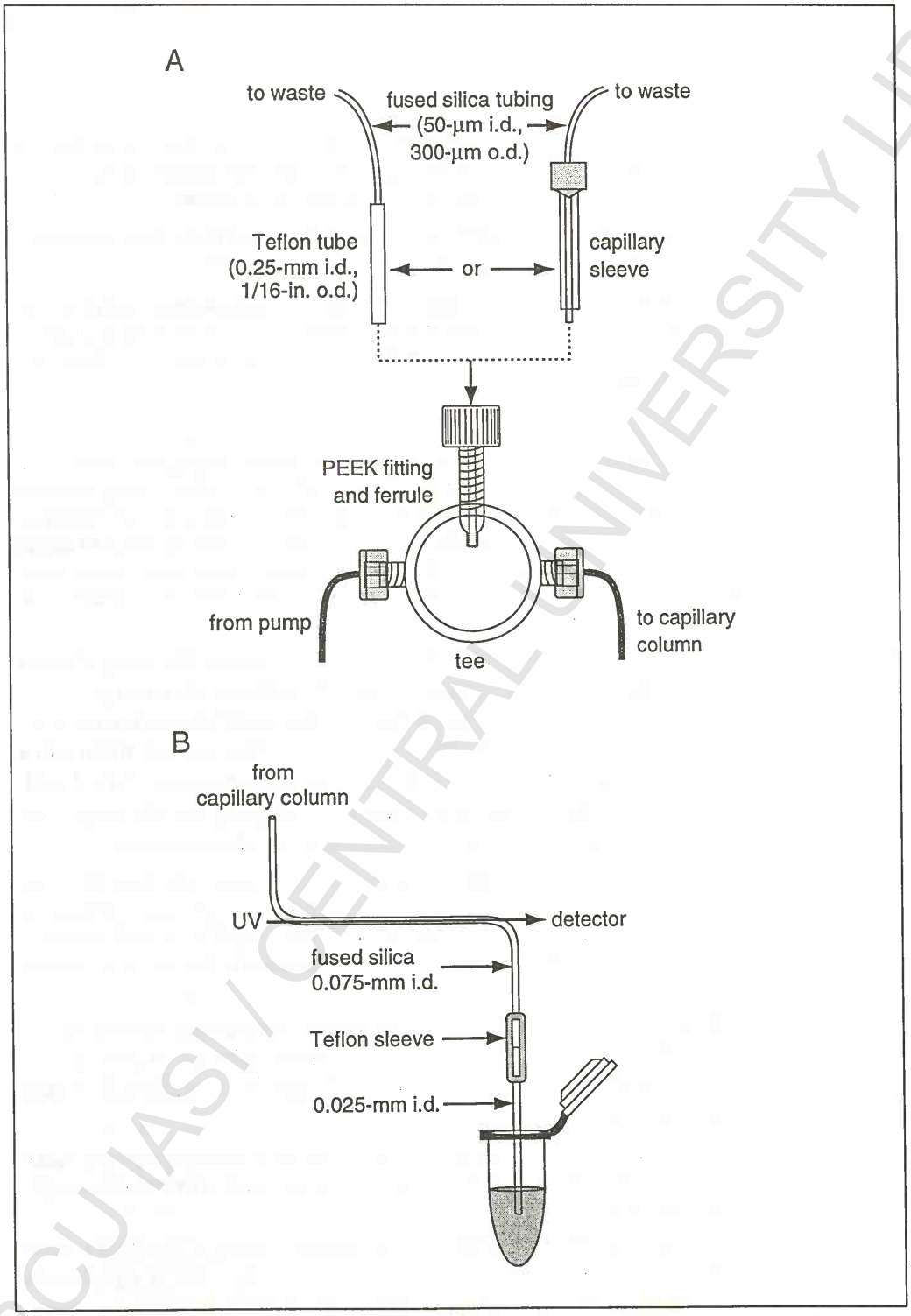

Figure 11.6.1 Modifications for capillary HPLC system. (**A**) Assembly of capillary fittings and splitter. (**B**) Modification of U or Z flow cell.

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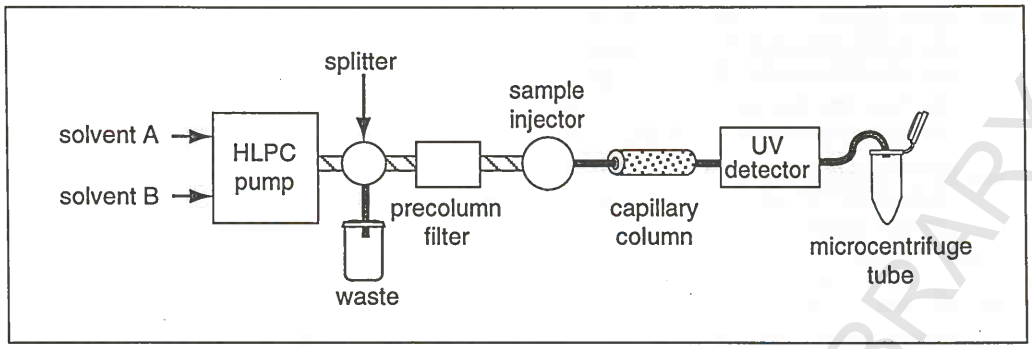

Figure 11.6.2 Capillary HPLC system.

mixer, which are removed to reduce the gradient delay caused by their large internal volumes. The high-pressure outlet of the mixing tee is connected directly to the splitter. Figure 11.6.2 shows a schematic diagram of the capillary HPLC system.

2. Connect outlet of splitter to injector and connect the injector to the in-line precolumn filter with 0.005-in.-i.d. PEEK tubing.

   The precolumn filter protects the capillary tubing and the column from clogging. The injector is fitted with a 20-µl sample loop. Any size sample loop can be used; however, a large loop will require a long wait for the sample to load on a 0.32-mm-i.d. column at a flow rate of 3 to 5 µl/min.

3a. Install capillary flow cell.

   The small peak volumes of capillary HPLC prevents the use of conventional flow cells. Instead, a length of capillary tubing with a short section of its polyamide coating removed is used as a flow cell. LC Packings has developed a capillary tube that is bent in the shape of a U or Z. The UV beam is aligned with the short axis of the U, increasing the path length of the flow cell. Although these flow cells have shorter path lengths than many conventional ones, the short path length and thin walls of the capillary tubing allow peptide detection at 195 nm.

3b. *Optional:* Modify the capillary flow cell (Fig. 11.6.1B) to reduce the delay volume by carefully cutting the 0.075-mm-i.d. outlet tubing of the flow cell, leaving ~1 to 2 cm adjacent to the Z cell (the fused silica tubing can be easily cut with a ceramic tubing cutter). Connect a 30-cm length of 0.025-mm-i.d., 0.280-mm-o.d. fused silica tubing with a 0.25-mm-i.d. Teflon sleeve to the remaining short outlet of the Z cell. The connection should be made inside the detector housing and the glass capillary tubing must be touching each other to avoid introducing any dead volume.

   This modification eliminates the need for two stopwatches for peak collection. The flow cell modification reduces the delay volume to 0.45 µl, corresponding to a delay of 6 sec for a flow rate of 3.5 µl/min. The short delay greatly facilitates hand collection of HPLC fractions and the small-i.d. outlet tubing provides backpressure that prevents bubble formation in the flow cell.

4. Connect the 0.32-mm-i.d. C18 capillary column to the sample prefilter with the 1/16-in. PEEK fitting supplied with the column. Connect outlet tubing (75-µm-i.d.) of capillary column to capillary flow cell with a short length of 0.25-mm-i.d. Teflon tubing, also supplied with the column.

   The 0.32-mm × 15-cm C18 column from LC Packings is the best general-purpose column for tryptic peptides. Keystone Scientific, Metachem Technologies, and Micro-Tech Scientific also sell capillary columns.

   The Teflon tubing slips over the ends of the capillary tubing, creating a zero-dead-volume union between the column outlet and the detector union. Resolution will be significantly diminished if a gap is present between the adjacent pieces of capillary tubing.

5. Prepare the capillary HPLC system for sample run (see Basic Protocol 2).

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PEPTIDE MAPPING BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION (MALDI) MASS SPECTROMETRY

Protein digests are mass analyzed either directly as an unfractionated mixture or as individual fractions from reversed-phase HPLC. The peptide-containing solution is mixed with a UV-absorbing matrix and applied to the sample stage of the mass spectrometer. Crystals of the peptide/matrix mixture form on the stage. This stage is inserted into the time-of-flight mass spectrometer vacuum system and irradiated with a UV laser to obtain a spectrum (see *UNITS 16.1 & UNIT 16.2*). Greater mass accuracy is achieved by addition of known peptide(s) as internal mass standards.

Materials

Peptide sample
30% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid (TFA)
MALDI matrix solution (see recipe)
Mass standard solution (see recipe)
Time-of-flight mass spectrometer (e.g., PerSeptive Biosystems–Vestec, Micromass, Shimadzu, Brucker, Finnigan MAT, or Hewlett-Packard)

1. Dilute samples in 30% acetonitrile/0.1% TFA to a final concentration of 0.05 to 5 pmol/µl.

   Salt and buffer concentrations should be <50 mM. Most detergents, especially SDS, are not tolerated. If necessary, reduce the ionic strength by dilution, HPLC, or size-exclusion chromatography (UNIT 8.3). If volatile buffers have been used, they may be removed by lyophilization and the dried sample redissolved in 2% TFA/50% acetonitrile.

2. Mix 2 µl of sample with 2 µl MALDI matrix solution in a 0.5-ml microcentrifuge tube.

3. Apply 1 to 2 µl of the mixture to the sample target, depending on the target size. Allow sample to dry completely and crystals to form without heating or applying vacuum.

   Alternatively, 0.5 µl of sample solution and 0.5 µl of matrix solution may be mixed directly on the target. The solutions should be mixed well by repeatedly drawing the liquid into a pipettor.

   The α-cyano-4-hydroxycinnamic acid (4HCCA) matrix solution works well for most peptides. An alternative is 2,5-dihydroxybenzoic acid (DHB), with or without either fucose or 5-methoxysalicylic acid additives. Another alternative is 3,5-dimethoxy-4-hydroxy-trans-cinnamic acid (sinapinic acid), but this produces a large background below 2000 Da and is not recommended for smaller peptides. Sinapinic acid or 4HCCA work best for larger peptides (>3000 Da). Due to preferential ionization of particular peptides in mixtures, it is advisable to obtain spectra for peptide mixtures with more than one matrix.

4. Insert sample into vacuum chamber of the mass spectrometer and follow the mass measurement procedure recommended by the instrument's manufacturer.

   Laser intensity (fluence) should be set just above the threshold for appearance of ion intensity. The observed signal (m/z) corresponds to $M+H^+$. Larger peptides may yield some $(M+2H)^{2+}$, which will appear at half the peptide mass (M/2). At high laser fluence, dimers may be formed as artifacts of the ionization process. Peptide mixtures may display preferential ionization for some peptides, and the relative peptide levels may vary from crystal to crystal. For this reason, spectra should be acquired from several different crystals on the target to obtain a representative display of peaks. The relative peak abundances in some cases (but not always!) give a semiquantitative image of the relative concentrations of peptides in the mixture, but they should not be relied upon for quantitation. Additional spectra acquired from the peptide mixture in a different matrix sometimes provide signals for peptides with suppressed ionization.

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5. Calibrate the mass spectrometer with a mixture of peptides of known mass. Obtain calibrant peptide mass spectra as described in steps 2 to 4. Calibrate the instrument according to the manufacturer's recommendation.

An equimolar solution (~1 pmol/μl of each component) of des-Arg-bradykinin (e.g., Sigma) and ACTH-CLIP (18-39) provides two peaks that cover the masses of most peptides. The centroid of the peak corresponds to the isotopically averaged mass and should be used for calibration and mass assignment. The average masses ($M+H^+$) for des-Arg-bradykinin (905.1 Da) and ACTH-CLIP (18-39) (2466.7 Da) are generally reliable as calibrants. When sufficient resolution is available, the monoisotopic peaks should be used for calibration: des-Arg-bradykinin 904.468, ACTH-CLIP (18-39) 2465.199. For higher mass peptides bovine insulin ($M+H^+$ = 5734.5) may be used for calibration. Mass accuracy is best when the calibration peaks are close in mass and abundance to the unknown sample being analyzed. The laser fluence should be constant for the calibrant and unknown sample spectra.

6a. *If >500 fmol of unknown peptide sample is available:* Mix an equimolar concentration of des-Arg-bradykinin/ACTH-CLIP (18-39) standard solution with the unknown sample. Repeat mass analysis of sample with internal mass standards as described in steps 2 to 4.

6b. *If no additional unknown peptide sample is available:* Redissolve sample and matrix remaining on target after analysis in step 4 with 1 μl of 2% TFA/50% acetonitrile. Add an equimolar concentration of mass standard solution. Allow mixed sample to dry on sample target and repeat mass analysis as described in step 4.

Internal mass calibration provides greater mass accuracy than external calibration. The amount of internal standard may need to be varied in several samples in order to obtain peak intensities that are approximately the same for the standard and the unknown sample. Avoid adding an excess of internal standard, which can suppress ionization of the unknown sample.

ALTERNATE PROTOCOL

PEPTIDE MAPPING BY MALDI MASS SPECTROMETRY USING THE MATRIX FAST-EVAPORATION METHOD

Preparation of a thin layer of matrix onto which the sample is applied offers advantages for small quantities of peptides and for contaminated samples. Matrix is applied to the sample stage in a highly volatile solvent that leaves a thin layer of matrix after evaporation. The uniform layer yields more uniform, intense signal across the sample. The sample is easily washed for efficient removal of contaminating salts and other buffer components that may interfere with ionization. Nitrocellulose is added to the matrix to provide greater peptide binding during the rinse step and to give greater structural support to the matrix, especially in the presence of higher concentrations of organic solvent that readily dissolve the matrix. This method is applicable to any type of MALDI instrument and gives superior results for a broad range of peptide and protein samples.

Additional Materials (also see Basic Protocol 3)

Fast-evaporation MALDI matrix solution (see recipe)
5% and 0.1% (v/v) trifluoracetic acid (TFA)

1. Apply 0.5 to 2.0 μl fast-evaporation MALDI matrix solution to a clean sample target.

The solution must be applied quickly due to the high volatility of the acetone. A uniform, white surface 3 to 5 mm in diameter will form in 3 to 5 sec.

2. Apply 0.3 to 2.0 μl peptide sample to target surface. Allow sample to dry completely.

The matrix dissolves easily in basic solution or in the presence of a high concentration of organic solvent (e.g., >30% acetonitrile). If the peptide sample is not already acidic, apply

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1.0 µl of 5% TFA to the target before applying sample. For samples that contain high concentrations of organic solvent, apply 1.0 µl of water to the target before applying the sample.

3. Rinse sample with 4.0 µl cold 0.1% TFA. After 10 sec, carefully remove the solution from the target surface with a pipet, or blow solution off target with a stream of nitrogen. Repeat rinse.

   Rinse step may be eliminated if the peptide sample is free of salts. If the salt content of the sample is unknown, try to obtain signal without rinsing. If the results are unsatisfactory, remove the sample from the mass spectrometer and perform the rinse steps.

4. Insert sample into vacuum chamber of the mass spectrometer and follow the mass measurement procedure recommended by the instrument's manufacturer (see Basic Protocol 3).

   Best results are obtained when samples are analyzed immediately. The authors have observed a decrease in signal intensity when samples are stored >24 hr. They have not observed any decrease in signal when samples are prepared according to Basic Protocol 3 using α-cyano-4–hydroxycinnamic acid (4HCCA).

5. Calibrate the mass spectrometer with a mixture of peptides of known mass (see Basic Protocol 3).

   Because the peptide sample often covers only a part of the matrix spot, a small volume (0.1 to 0.3 µl) of the mass standard solution may be applied to the side of the matrix spot (after the peptide sample is dried on the other side of the matrix spot and rinsed) to provide a pseudo-internal standard. The mass spectrum of this small calibration sample may be taken immediately before or after the peptide spectrum to provide an external calibration with high accuracy. Alternatively, the signal from this sample may be included in the sample spectrum to provide internal calibration without the risk of ion suppression by the calibration compounds.

CAPILLARY ELECTROPHORESIS ANALYSIS

BASIC PROTOCOL 4

Capillary electrophoresis (also see *UNIT 10.9*) separates peptides by electrophoretic migration and electroosmotic flow using a high-voltage electric field in a fused silica capillary. The high voltage (20 to 25 kV) and the small internal volume of the fused silica capillary provide resolution that often exceeds 100,000 plates/meter. This high resolution combined with a different separation mechanism will often provide baseline resolution of peaks that coeluted during a reversed-phase HPLC separation. Very small volumes are required (10 to 200 nl), allowing the majority of the sample to be used for other methods of analysis or, if necessary, for repurification.

Materials

0.1 N NaOH
Peptide sample
20 mM sodium phosphate, pH 2.5

Capillary electrophoresis (CE) instrument
75-µm-i.d. uncoated capillary column with separating length of 50 cm (from manufacturer of CE instrument or Polymicro Technologies)

1. Set detector wavelength to 200 nm.

   If excessive baseline noise occurs at 200 nm, increase wavelength to 214 nm.

2. Wash capillary with 0.1 N NaOH for 1 min, followed by Milli-Q water for 1 to 2 min.

3. Equilibrate capillary with 20 mM sodium phosphate buffer, pH 2.5, for 5 min or until a stable baseline is established.

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4. Inject sample using a pressure or vacuum injection of 5 to 30 sec.

   Some instruments inject the sample by creating a vacuum, whereas others apply a gas pressure to the sample reservoir. Both methods are capable of producing reproducible injections. Other instruments employ electrokinetic injection, in which an electric field is used to move the sample into the separating capillary. A drawback to this procedure is that the velocity of sample migration is dependent on the charge of the sample. This can result in a disproportionate injection of the sample components that have a higher charge. Also see UNIT 10.9.

   The peptide sample does not generally require any special preparation before injection.

5. Separate the components of the sample at 20 to 25 kV.
6. Repeat steps 2 to 5 for each additional sample. Replace capillary inlet and outlet buffer reservoir with fresh 20 mM sodium phosphate after every five runs.

   Buffer ion depletion can affect the reproducibility of the separation.

REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2E**; for suppliers, see **SUPPLIERS APPENDIX**.*

Detector flow cell

A flow cell with an internal volume <12 µl is required for 2-mm columns and with internal volume <5 µl for 1-mm columns. Many of the newer HPLC systems designed for 2-mm columns are supplied with flow cells that have appropriately small (0.005-in.-i.d.) outlet tubing that allows peaks to be collected without loss of resolution caused by mixing. Older instruments designed only for 4.6-mm-i.d. columns, such as the Hewlett-Packard HP-1090, may need to be modified. This can easily be accomplished for most instruments by connecting 75-µm-i.d. FSC tubing (Polymicro Technologies) or 0.005-in.-i.d. PEEK tubing (Upchurch Scientific) to the outlet of the flow cell. This tubing should be kept as short as possible to prevent loss of resolution from mixing and to minimize backpressure on the flow cell (excessive back-pressure can result in a broken flow cell).

Fast-evaporation MALDI matrix solution

5 mg nitrocellulose
20 mg α-cyano-4-hydroxycinnamic acid (4HCCA)

Dissolve nitrocellulose and 4HCCA in 500 µl acetone in a 1.5-ml microcentrifuge tube. Add 500 µl isopropanol and mix completely. Store at –20°C 1 to 2 months. Keep the cap tightly sealed at all times—evaporation of the solvent is the main problem encountered in repeated use.

Before adding isopropanol, it may be necessary to vortex the material for 1 to 2 min to dissolve the nitrocellulose completely.

Use only pure nitrocellulose (e.g., from Schleicher & Schuell). Some nitrocellulose membranes contain other material supports.

MALDI matrix solution

Prepare in 30% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid either:

1. 20 mg/ml α-cyano-4-hydroxycinnamic acid (use only the supernatant as matrix solution);
2. 50 mg/ml 2,5-dihydroxybenzoic acid (DHB); *or*
3. Saturated solution of 3,5-dimethoxy-4-hydroxy-*trans*-cinnamic acid (sinapinic acid; use only the supernatant as matrix solution).

Trifluoroacetic acid from Pierce is recommended because some manufacturers add antioxidants that can generate artifacts.

Alternatively, premade matrix solutions ("MALDI-grade") are also available from Hewlett-Packard.

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Mass standard solution

Dissolve 1 pmol/ml des-Arg-bradykinin (e.g., Sigma) and 2 pmol/ml ACTH-CLIP (18-39) in 30% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid (Pierce).

COMMENTARY

Background Information

Reversed-phase HPLC has been widely utilized for analysis and preparative fractionation of peptides. Although many different buffers have been described in the literature, trifluoroacetic acid (TFA) has become the most widely used ion-pairing agent for reversed-phase chromatography of peptides. The volatility of TFA enables it to be compatible with many analytical methods including protein sequencing, online electrospray mass spectrometry, amino acid analysis, and capillary electrophoresis.

The development of columns with small internal diameters (2 and 1 mm) has greatly facilitated the ease of handling of small amounts (<200 pmol) of proteolytic digests of proteins (see Basic Protocol 1; Schlabach and Wilson, 1987). The use of 2-mm-i.d. columns generally results in resolution equivalent or superior to that obtained on a 4.6-mm-i.d. column, with significant cost savings in solvent purchase and disposal. Another advantage of smaller-i.d. columns is the increase in sensitivity they provide. The increase in detector signal is a function of the inverse square of the column radius. Reducing the column diameter from 4.6 to 1 mm results in a signal that is 20 times larger.

Microbore 1-mm-i.d. columns have been more problematic than 2-mm-i.d. columns. The inability of many HPLC pumps to deliver samples at a rate of 50 to 100 µl/min reliably has prevented widespread use of these smaller columns. Many commercially packed 1-mm-i.d. columns have had low theoretical plate counts compared to 2-mm-i.d. columns. This is primarily due to difficulty in packing these columns in small stainless steel tubes.

The problems associated with packing 1-mm stainless steel columns are generally not observed with columns of diameters <1 mm. These columns are packed into fused silica capillaries that have extremely smooth walls compared to stainless steel tubing. Capillary columns have a high theoretical plate count that often exceeds 100,000 plates per meter, resulting in higher resolution than can usually be achieved in a 1- or 2-mm-i.d. column. The high resolution and small internal diameters of capillary columns are ideal for high-sensitivity peptide mapping at the 5-pmol level and below (see Basic Protocol 2; Cobb and Novotny, 1989; Davis and Lee, 1992). Capillary columns can be fabricated in the laboratory using a slurry packing technique with an HPLC pump (Moritz and Simpson, 1992). Many different types and sizes of capillary columns are commercially available. Capillary columns of 0.8-mm i.d. can be used on some HPLC systems without using a splitter. These HPLC systems must be capable of delivering a flow rate of 25 to 50 µl/min and be equipped with a micro flow cell (<1 µl) to prevent a loss of resolution caused by mixing in the flow cell. The short path-length of these flow cells can often outweigh any gain in sensitivity.

Replacing a micro flow cell with a 200-µm-i.d. U-shaped capillary cell can increase detector sensitivity. This type of capillary cell is available for a variety of UV detectors from LC Packings. An 0.32-mm-i.d. capillary column can provide high sensitivity and good recovery for analytical and micropreparative collection of peptides from in situ digests of proteins electroblotted on one- and two-dimensional gels (*UNIT 10.7*; Wong et al., 1993). An 0.32-mm-i.d. capillary column requires a capillary flow cell to prevent loss of resolution from mixing.

Although capillary flow cells typically have short path lengths, the thin walls of the capillary and the short path length of the detector cell allow transmission of UV light at 195 nm. The shorter wavelength allows higher-sensitivity peptide detection than can be obtained at 214 nm.

Peak collection of capillary HPLC fractions can be tedious. However, a significant advantage to this method is the high concentration present in the collection tube as a result of the small peak volumes (0.5 to 3 µl). The high concentration in the sample tube minimizes sample loss by adsorption to the tube walls. When a 5-pmol sample is diluted to 100 to 200 µl—the typical peak volume obtained from a separation on a 1- to 2-mm column—adsorption to the tube walls may occur.

Despite the small internal volume of capillary columns, the capacity can be relatively large (>1 nmol). Large-volume injections (>1 ml) may require a wait of several hours for the sample to load on the column. The increased volume will not have a significant effect on resolution for peptide separations, however, because of the absorption/desorption mecha-

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nism of peptide retention. Another advantage of capillary HPLC is its compatibility with on-line electrospray ionization mass spectrometry (*UNIT 16.1*), a result of the low flow rates (3 to 5 µl/min) employed with these columns. On-line electrospray ionization mass spectrometry allows mass measurement of each component that elutes from the column and in some cases provides sequence information (Griffin et al., 1991; Huang and Henion, 1991; Hess et al., 1993).

Small-i.d. columns can provide high-resolution separations, but some peaks may still contain multiple components. MALDI mass spectrometry (see Basic Protocol 3) and capillary electrophoresis (see Basic Protocol 4) are rapid methods for analyzing peak purity. MALDI mass spectrometry (Hillenkamp et al., 1991; Beavis and Chait, 1996; *UNIT 16.2*) is capable of analyzing low-femtomole quantities of peptide mixtures or isolated peptide fractions (Billeci and Stults, 1993). For example, an in situ tryptic digest of 2 to 5 pmol of a PVDF-electroblotted protein may yield 1- to 2-pmol peptide fractions after enzymatic digestion and separation on a capillary HPLC column. Only a small aliquot (1% to 10%) of a 1-pmol peptide fraction is usually required to determine both purity and molecular weight by MALDI mass spectrometry (Henzel et al., 1993). This information can be useful in deciding which peptide fraction to sequence as well as deciding on the number of cycles with which to program the sequencer. The peptide mass is also indispensable for corroborating sequence data and identifying post-translational modifications (Geromanos et al., 1994).

Capillary electrophoresis (CE; *UNIT 10.9*) can also provide an indication of peak purity. The electropherogram (chromatogram) produced by the CE instrument can be used to approximate the degree of purity of peptide fractions. This is in contrast to MALDI mass spectrometry, which is not quantitative and in which two components with the same concentration may result in significantly different signals. CE generally requires a peptide concentration of 10 pmol/µl, which is 2 to 3 orders of magnitude larger than that required by MALDI mass spectrometry. Although some progress has been made in injecting larger volumes by concentrating the sample within the CE column (Aebersold and Morrison, 1990; Chien and Burgi, 1992; Figeys et al., 1996; Tomlinson et al., 1996), CE injection volumes are generally limited to <50 nl.

Critical Parameters

Selection of stationary phase

A large variety of reversed-phase stationary phases have been developed. The most commonly employed packings for peptide separation are based on porous silica derivatized with C4, C8, or C18. These columns are available with pore sizes ranging from 100 to 4000 Å. Columns of small particles containing small pores generally provide higher resolution than columns made of materials with larger pores, but peptide recovery is often lower. Peptides obtained from trypsin, chymotrypsin, or pepsin digests, which are often short (<15 residues), are preferably separated on a C18 column. Peptides from Lys-C and cyanogen bromide cleavages, which are often >15 residues long, are usually separated on a C4 column with 300- or 1000-Å pores.

Selection of mobile-phase solvent

Another important variable is the choice of the mobile phase. Mixtures of trifluoroacetic acid (TFA) in water with either acetonitrile or propanol as the organic solvent are the most widely utilized types of mobile phase for peptide separation. The choice of acetonitrile or propanol can affect peptide recovery and resolution. Acetonitrile will generally result in greater resolution than propanol; however, propanol usually provides higher peptide recovery. Complex peptide separations resulting from digestion of large proteins (>100 kDa) demand high-resolution separations and hence are best done using acetonitrile.

Increasing the length of the gradient can significantly increase resolution. A tryptic digest of a 100-kDa protein may require a 2- to 3-hr gradient. Peptides that fail to resolve in the course of an extended gradient can often be separated by repeating the chromatography using a different column (C4 or C8 in place of C18). The selectivity of the separation also can be affected by substituting a different organic solvent (i.e., propanol in place of acetonitrile) or by changing the pH of the separation. Rechromatography on the same column using a different organic modifier will often resolve coeluting peaks. Rechromatography can be repeated as many times as necessary but at the expense of some losses with each step. The sample must be diluted with water or concentrated in a Speedvac to lower the organic solvent concentration prior to rechromatography in order to allow the peptides to adsorb to the re-

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versed-phase column. Figure 11.6.3 shows the relationship of pore size and alkyl chain length to resolution and peptide recovery.

Sample preparation and storage

Peptide fractions should be stored at 5°C in polypropylene tubes, not in polystyrene or glass. Prolonged storage can result in some evaporation of organic solvent from the sample, which may lead to sample adsorption to the microcentrifuge tube. Adding neat TFA to the sample tube (33% final concentration) can resolubilize peptides that may have precipitated or adsorbed to the tube walls (Erdjument-Bromage et al., 1993).

Solubility of peptides for MALDI can also be enhanced by addition of up to 50% acetonitrile and 2% TFA. Alternatively, up to 1% octyl-β-glucoside may assist in solubilization and is compatible with MALDI (Gharahdaghi et al., 1996). As fractions isolated by RP-HPLC with a volatile mobile phase are normally quite clean, no further cleanup of the sample is usually needed before analysis by MALDI. Preparation of samples for MALDI is easily done by taking a 0.3- to 0.6-µl aliquot of an HPLC fraction straight from the fraction collection tube and mixing it with matrix directly on the sample target.

Mass measurement of unfractionated proteolytic digests is useful for rapidly confirming the sequence of known proteins (Billeci and Stults, 1993), for identifying proteins by searching a sequence database with peptide masses (Henzel et al., 1993), and for assessing completeness of digestion. The digestion is preferably performed in a volatile buffer, such as ammonium bicarbonate, ammonium acetate, or *N*-ethylmorpholine. The buffer should be removed by vacuum centrifugation and the peptides solubilized in 50% acetonitrile/2% TFA. If nonvolatile buffer components are used, they must be diluted below 50 mM. Preferably, samples can be washed after application when the fast-evaporation matrix is used (Shevchenko et al., 1996; see Alternate Protocol). Analysis of subpicomole amounts of peptide may only be successful at substantially lower buffer concentrations. Additional details on sample preparation are found in *UNITS 16.1 & UNIT 16.2*.

Special consideration for MALDI and CE

Mass accuracy for peptide measurement (1- to 3-kDa peptides) with a linear time-of-flight instrument is typically ±1 to 2 Da with external calibration of the mass axis. Higher mass accuracy is achieved by measuring the sample with an internal mass standard, usually a pair of peptides of known mass with signal intensity approximately the same as the unknown sample. Repeated experiments may be necessary to achieve the proper ratio of standard to sample. With proper internal mass standards and an adequate signal-to-noise ratio, the mass accuracy can be ±0.01% (±0.2 Da at *m/z* 2000). Higher mass accuracy and resolution may be obtained with reflector instruments (*UNIT 16.1*) or delayed extraction (Vestal et al., 1995).

The main variable in capillary electrophoresis of peptides is the choice of the separation buffer. Peptides that comigrate can often be separated by changing the pH of the buffer (Grossman et al., 1988). Changing the pH from 2.5 to 7.0 will often have a major effect on the separation. The ionic strength of the buffer can be increased for peptides that have slow migration rates. A different buffer can be tried when pH and ionic strength fail to improve the separation. Buffer additives can also be utilized to further enhance selectivity (Cobb and Novotny, 1992).

Troubleshooting

One of the main problems associated with reversed-phase HPLC is the occurrence of a

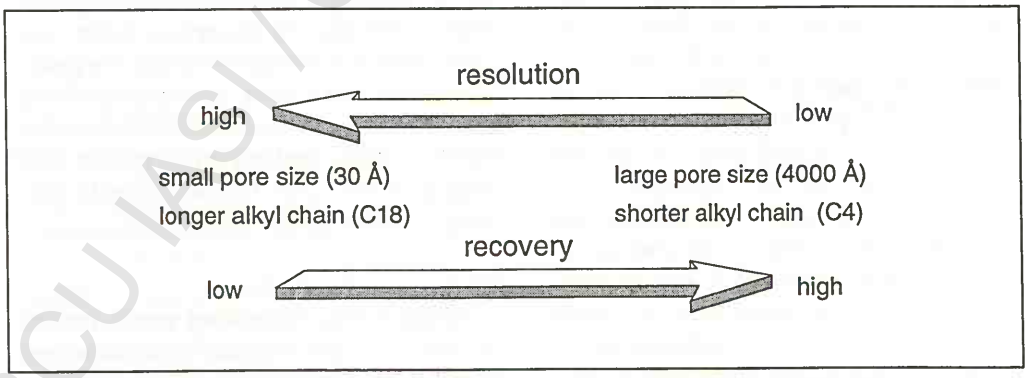

Figure 11.6.3 Effects of pore size and alkyl chain length on the resolution and recovery of peptides.

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drifting baseline. This is usually the result of contaminants in the mobile phase or inadequate mobile phase degassing. It is essential to use high-purity HPLC reagents and Milli-Q-quality water and to wear powder-free gloves for all manipulations. A baseline rise that increases with increasing organic modifier (solvent B) can be corrected by adjusting the amount of TFA added to the organic solvent. Titrating the amount of TFA in the solvents is especially important for capillary HPLC. In general a concentration of 0.05% to 0.08% TFA in solvent B will usually result in an acceptable baseline.

Baseline artifacts, which may appear as negative fluctuations in the baseline, can be caused by a number of HPLC pump malfunctions, including leaks (Dolan, 1991). It is essential to keep HPLC pumps in optimum condition for high-sensitivity HPLC. It is sometimes difficult to detect minor leaks, which can cause a major baseline disturbance during use of capillary HPLC columns. These leaks can usually be detected by placing a dry Kimwipe on the suspected fitting.

Occasionally the reversed-phase column can become contaminated by the sample, causing ghost peaks to appear. These are peaks that appear in blank runs when a gradient is run without injecting a sample. Sometimes several blank runs may be necessary before the ghost peaks completely disappear. Extended washing with 80% to 100% organic modifier can also be tried. If both tactics have been tried without success, the column should be replaced.

Increased backpressure (>500 p.s.i.) above the normal backpressure of the column may indicate a clogged frit or a contaminated column. Use of a precolumn filter can prevent the column frit from clogging. The precolumn filter is easily replaced and relatively inexpensive. Column inlet frits can be replaced, but care must be exercised to prevent loss of column packing material. Capillary columns are particularly prone to clogging and should always be used in conjunction with a precolumn filter.

Peptide standards have been developed to monitor HPLC performance. Commercial standards (e.g., PE Applied Biosystems) should be run when a new column is first utilized and when resolution appears to deteriorate (Mant and Hodges, 1990). Alternatively, a tryptic digest of a protein that results in well-resolved peptides on reversed-phase chromatography can be utilized to monitor column resolution. The digest should be solubilized in 0.1% TFA and stored frozen at –70°C between uses.

Reversed-phase columns should be stored in organic solvent that does not contain TFA, as TFA can slowly dissolve silica. Capillary columns will last significantly longer if this practice is followed.

Unsuccessful mass measurement by MALDI may be due to insufficient sample (<10 to 50 fmol). Sample loss is often a result of poor solubility of the peptide or the presence of contaminants that suppress ionization or crystal formation. Peptide solubility is aided by addition of acid, organic solvent, or octyl-β-glucoside. Alternative solvents such as formic acid or methanol may also assist in solubility and ionization (Cohen and Chait, 1996). In the case of an unfractionated digest or a nonvolatile buffer system, incompatible buffer components may be accommodated simply by diluting the sample, if the peptide concentration permits. Alternatively, salts and buffers are often excluded from the less soluble peptide/matrix crystal, so a brief (~5-sec) wash of the sample target with cold water may remove many of the interfering salts.

CE instruments are relatively simple and are less problematic than HPLC equipment. The two major problems in CE are clogging of the capillary tubing and artifacts resulting from reagent or sample contaminants. Complete absence of current flow usually indicates a blocked capillary. The clog is often found on the sample inlet side and can be usually corrected by removing a short section (2 to 5 mm) of capillary from the column inlet end. The high sensitivity of CE requires high-purity reagents. A number of chemical distributors now sell CE-purity regents.

Anticipated Results

Reversed-phase HPLC should result in resolution and good recovery of the majority of the peptides (<30 residues) present in a typical proteolytic digest of a protein (<30 kDa). The use of capillary HPLC for tryptic peptides should allow recovery of peptides at the 1- to 2-pmol level. CE should be capable of separating peptides that coelute as a single peak on HPLC; however, the concentration must be at least 10 pmol/μl. Peptides in the concentration range of 10 fmol/μl to 50 pmol/μl can be analyzed by MALDI mass spectrometry.

Time Considerations

Reversed-phase separations usually require 30 min to 1 hr per analysis. When additional blank gradients are run, several hours may be required. CE analysis of a purified peptide

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fraction may require from 15 to 60 min, depending on the charge and mass of the sample and the buffer utilized. Mass analysis by MALDI requires 1 to 2 min for preparation of each sample target, 5 to 10 min drying time, and 2 to 5 min per sample for data acquisition. Data processing may add an additional 5 to 10 min.

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A comprehensive guide to HPLC of peptides and proteins

Contributed by William J. Henzel and John T. Stults
Genentech, Inc.
South San Francisco, California

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Removal of N-Terminal Blocking Groups from Proteins

Two enzymatic methods are commonly used in N-terminal sequence analysis of blocked proteins, one for N^{α}-pyrrolidone carboxyl-proteins in solution (Basic Protocol 1) or blotted onto a membrane (Alternate Protocol 1) and the other for N^{α}-acyl-proteins blocked with other acyl groups (Basic Protocol 2, Alternate Protocol 2). The enzyme involved in the first of these reactions, pyroglutamate aminopeptidase (EC 3.4.19.3), works on intact proteins as well as on peptides, and represents an excellent reagent in the unblocking/sequencing arsenal. The Support Protocol describes a colorimetric assay for pyroglutamate aminopeptidase activity. The enzyme used for unblocking N-terminal acetyl/formyl amino acids, acylaminoacyl-peptide hydrolase (EC 3.4.19.1; also referred to as acyl-peptide hydrolase, *N*-acetylalanine aminopeptidase, and acylamino acid–releasing enzyme in the literature) is more restricted in that it cannot use intact proteins as substrate, but can be used to obtain N-terminal sequence from peptides containing up to 20 residues. Consequently, any sequencing protocol with this latter enzyme must include fragmentation of the protein before unblocking can be carried out. To avoid extensive purification after fragmentation, newly generated peptides are chemically blocked with either succinic anhydride (Basic Protocol 2) or phenylisothiocyanate/performic acid (Alternate Protocol 2), and hydrolase is applied to the total mixture of peptides, only one of which, the acylated N-terminal peptide, should be a substrate for hydrolase. After incubation, the mixture of peptides is subjected to sequence analysis.

A great variety of chemical methods have been applied to the problem of unblocking N-terminally blocked proteins. The methods include acid-catalyzed hydrolysis (Basic Protocol 3) or methanolysis, hydrazinolysis, and β-elimination after acid-catalyzed N-O shift. For each of these methods, a variety of conditions have been adapted to satisfy the unique properties of different blocking groups and different proteins/peptides. For chemical removal of acetyl and longer-chain alkanoyl groups (Alternate Protocol 3), conditions may have to be harsh enough to cause extensive cleavage of, for example, aspartyl peptide bonds. For chemical removal of formyl groups and opening of the cyclic imide of pyrrolidone carboxylate (Alternate Protocol 4), reaction conditions can generally be sufficiently mild that few or no peptide bonds are cleaved. Blocked proteins that are devoid of aspartate and other labile peptide bonds are not often encountered, so the best substrates for chemical unblocking methods are aspartate-free N-terminal peptides. Because the yield of unblocked peptide varies extensively, the amounts of protein recommended for these protocols are considerably larger than those needed for the direct sequencing of an unblocked protein.

It is important to keep in mind that the side chain amides of asparagine and glutamine are among the sensitive amide bonds that are likely to be modified by the various treatments. In general, it should be safe to conclude that even low yields of amide together with high yields of the acid originated as the amide in the acid-treated sequence under study. However, if hydrolysis is extensive enough that the amide cannot be observed at all, the wrong amino acid will obviously be identified.

The choice of method for removing N-terminal blocking groups depends on the nature of the blocking group and the N-terminal amino acid. In some cases information about the groups will be available from the prior history of the sample, from mass analysis, or from studies of homologous proteins. In the absence of such information, the process is trial and error. Testing for hydrazine lability, acid lability, and enzyme susceptibility provides clues as to the nature of the blocking group. Successful removal of the group should make it possible to obtain sequence information beyond the blocked residue.

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REMOVAL OF PYRROLIDONE CARBOXYLIC ACID WITH PYROGLUTAMATE AMINOPEPTIDASE TREATMENT OF PROTEINS IN SOLUTION

Pyrrolidone carboxylic acid (PCA), also called pyroglutamic acid (see Fig. 11.7.1), is formed by cyclization of glutamine or glutamic acid to form an amide bond between the α-amino group and the δ-carboxyl group. PCA is found on many naturally occurring proteins and peptides. In addition, PCA may be formed by cyclization of amino-terminal glutamine residues during protein isolation, proteolysis and separation of peptides, and/or protein sequencing (Crimmins et al., 1988). The presence of PCA precludes direct amino-terminal sequence analysis because the α-amino group is not available for reaction with the Edman reagent. The enzyme pyroglutamate aminopeptidase (EC 3.4.19.3) can be used to remove pyroglutamate and leave a free α-amino group on the adjacent residue accessible for Edman degradation and other chemical reactions. The enzyme may be used to digest proteins and peptides either in solution or after electroblotting to membranes (see Alternate Protocol 1).

The following protocol describes enzymatic removal of pyrrolidone carboxylic acid from proteins or peptides in solution. The reaction is generally more efficient in solution than on electroblotted proteins (see Alternate Protocol 1).

Materials

Protein or peptide, reduced and alkylated (*UNIT 11.1*)
PGA digestion buffer (see recipe)
Calf liver pyroglutamate aminopeptidase (EC 3.4.19.3; sequencing grade, Boehringer Mannhein)
0.05 M acetic acid
Nitrogen (research grade)

Screw-cap vial
Magnetic stirring plate

Additional reagents and equipment for dialysis (*UNIT 4.4 & APPENDIX 3B*)

1. Prepare an 0.5 to 1 mg/ml solution of protein or peptide in PGA digestion buffer.

   Before PGA digestion, the protein should be reduced and alkylated (UNIT 11.1). If the protein preparation contains components other than those specified for PGA digestion buffer, the protein solution should be dialyzed against PGA digestion buffer at 4°C (UNIT 4.4 & APPENDIX 3B) or otherwise desalted.

2. Transfer the protein solution to a screw-cap vial containing a stir bar.

   The vial should have a volume no greater than three times the volume of the sample.

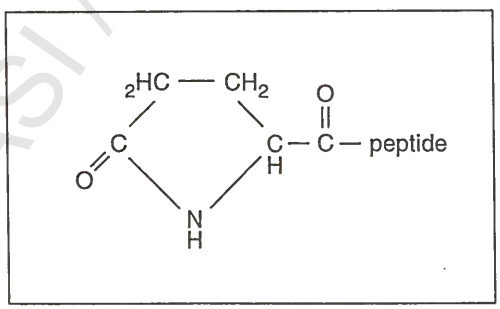

Figure 11.7.1 Pyrrolidone carboxylic acid in peptide linkage.

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3. Dissolve an aliquot of calf liver pyroglutamate aminopeptidase in water to give a concentration of 0.25 mg/ml (enzyme stock solution).

Sequencing-grade enzyme is supplied by the manufacturer (Boehringer Mannheim) in vials containing 25 μg enzyme protein. The lyophilized material supplied by the manufacturer contains ~70% (w/w) sucrose, 17% (w/w) potassium phosphate, 8% (w/w) EDTA, and 5% (w/w) enzyme. A single vial should be reconstituted in 0.1 ml water, then flushed with nitrogen and stored ≤1 week at 4°C.

If bulk enzyme (Boehringer Mannheim) is used, reconstitute at 0.25 mg/ml. Some authors report successful storage of this preparation up to 1 month at –20°C in screw-cap vials that have been flushed with nitrogen.

4. Add sufficient enzyme stock solution to the protein solution in the screw-cap vial to give an enzyme/substrate ratio of ~1/300 (w/w). Flush the vial with nitrogen and stir the solution 7 to 9 hr at 4°C.

Enzyme/substrate ratio and digestion time are dependent on the particular protein or peptide being digested. The rate of removal of PCA varies with the adjacent residue (see Background Information).

Small peptides may be digested at higher temperatures (40°C) for shorter times, generally 2 to 3 hr (Dimaline and Reeve, 1983).

5. Add a second aliquot of enzyme stock solution (equal in volume to that added in step 4). Flush the vial with nitrogen and stir the solution an additional 14 to 16 hr at room temperature.

6. Dialyze the reaction mixture against 0.05 M acetic acid (*UNIT 4.4 & APPENDIX 3B*). The protein is now suitable for sequence analysis.

Alternatively, the protein or peptide may be separated from the reagents by any suitable chromatographic or electrophoretic technique (see Chapters 8 and 10). The reaction mix may also be applied directly to the sequencer support.

REMOVAL OF PYRROLIDONE CARBOXYLIC ACID WITH PYROGLUTAMATE AMINOPEPTIDASE TREATMENT OF ELECTROBLOTTED PROTEINS

ALTERNATE PROTOCOL 1

Identification of proteins separated by gel electrophoresis is conveniently achieved by performing amino-terminal sequence analysis after electroblotting the proteins onto polyvinylidene difluoride (PVDF) membranes (*UNITS 10.7 & 11.3*). If no sequence is obtained when the blotted protein is sequenced, the amino terminus of the protein may be blocked by pyrrolidone carboxylic acid (PCA) or another modification of the α-amino group. Digestion with pyroglutamate aminopeptidase (EC 3.4.19.3) is useful for removing PCA prior to sequencing.

Additional Materials (also see Basic Protocol 1)

Single protein band on PVDF membrane (*UNIT 10.7*) containing ≥50 pmol protein
0.5% (w/v) polyvinylpyrrolidone (mol. wt. 40,000, PVP-40; Sigma) in 0.1 M acetic acid
PGA digestion buffer (see recipe)
Screw-cap polypropylene microcentrifuge tube

1. Dissolve an aliquot of calf liver pyroglutamate aminopeptidase in water to give a concentration of 0.25 mg/ml (enzyme stock solution).

Sequencing-grade enzyme is supplied by the manufacturer (Boehringer Mannheim) in vials containing 25 μg of enzyme protein. The lyophilized material supplied by the

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manufacturer contains ~70% (w/w) sucrose, 17% (w/w) potassium phosphate, 8% (w/w) EDTA, and 5% (w/w) enzyme. A single vial should be reconstituted in 0.1 ml water, then flushed with nitrogen and stored ≤ 1 week at 4°C.

If bulk enzyme (Boehringer Mannheim) is used, reconstitute at 0.25 mg/ml. Some authors report successful storage of this preparation up to 1 month at –20°C in screw-cap vials that have been flushed with nitrogen.

2. Place single protein band on PVDF membrane in a screw-cap polypropylene microcentrifuge tube and rinse well with water. Add 0.5% PVP-40 in 0.1 M acetic acid to cover. Incubate 30 min at 37°C.

   After rinsing, as much as possible should be drained off without allowing the membrane to dry.

3. Rinse ten times with water.

4. Cover the PVDF membrane with PGA digestion buffer.

5. Estimate the amount (micrograms) of blotted protein present.

   The amount of protein present in the band may be estimated from the amount applied to the gel, or, preferably, by comparing the intensity of the stained band with a dilution series of a known protein. An estimate +/–100% is generally adequate.

6. Add enzyme stock solution to achieve an enzyme/substrate ratio of 1/20 (w/w). Flush the vial with nitrogen and close. Incubate 5 hr at 4°C, then 18 hr at room temperature.

7. Rinse the PVDF membrane with water several times. Air dry. The pieces are now suitable for sequence analysis.

SUPPORT PROTOCOL

COLORIMETRIC ASSAY FOR PYROGLUTAMATE AMINOPEPTIDASE ACTIVITY

Pyroglutamate aminopeptidase activity is not particularly stable. One cause of failure to remove an amino-terminal blocking group may be lack of activity in the enzyme stock solution. The following protocol describes the method used by Boehringer Mannheim and several authors for monitoring enzyme activity based on generation of β-naphthylamine from L-pyroglutamyl-β-naphthylamide. An alternative method for monitoring enzyme activity—assaying for the hydrolysis of a synthetic peptide with ninhydrin—is described in Doolittle (1972).

Materials

PGA digestion buffer (see recipe)
PGA-N substrate solution (see recipe)
25% (v/v) trichloroacetic acid
0.1% (w/v) sodium nitrite
0.5% (w/v) ammonium sulfamate
NED solution (see recipe)

13 × 100–mm test tubes
37°C incubator or water bath

1. Prepare a 13 × 100–mm test tube for each enzyme sample and a "no enzyme" blank. Add 1.0 ml PGA digestion buffer and 0.1 ml PGA-N substrate. Incubate 5 min at 37°C.

   The no enzyme blank is prepared identically to the enzyme sample, except that the enzyme solution is omitted from the incubation; it is added after the reaction has been stopped.

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2. Add 0.1 ml enzyme stock solution to enzyme sample tube; add nothing to blank tube. Mix well. Incubate exactly 15 min at 37°C.
3. Add 1.0 ml of 25% trichloroacetic acid to all tubes. Add 0.1 ml enzyme stock solution to blank tube. Mix well.

   If precipitate forms, filter through medium-grade filter paper into clean tubes.
4. Transfer 1.0 ml from each tube into a clean tube.
5. Add 1.0 ml of 0.1% sodium nitrite and mix well. Incubate 3 min at 37°C.
6. Add 1.0 ml of 0.5% ammonium sulfamate and mix well. Let stand ~5 min at room temperature.
7. Add 2.0 ml NED solution and mix well. Incubate 20 min at 37°C.
8. Mix samples and read absorption at 578 nm.
9. Use A_{578} to calculate activity (U/ml enzyme solution) using the following formulas:

$$\Delta A_{578} = A_{578,\text{sample}} - A_{578,\text{blank}}$$

$$\text{Activity} = 0.163 \times \Delta A_{578}$$

REMOVAL OF ACYLAMINO ACIDS BY HYDROLASE TREATMENT USING SUCCINIC ANHYDRIDE AS BLOCKING REAGENT

BASIC PROTOCOL 2

Instead of purifying the N-terminal peptide after fragmentation of a blocked protein, the generated peptides can be rendered inert by chemical blocking using one of two blocking reactions: succinylation with succinic anhydride (as in this protocol) and carbamoylation by reaction with isothiocyanate followed by oxidation with performic acid to convert thiocarbamate to carbamate (Alternate Protocol 2). The general strategy for establishing N-terminal sequences in N^{α}-acylated proteins is outlined in Figure 11.7.2. All steps—fragmentation of the blocked protein, blocking the newly generated N-termini, treatment with aminoacyl-peptide hydrolase and finally with acylase—are performed in a single test tube to ensure optimum yield. Because the enzyme releases an N-terminal acylamino acid, special considerations must be given to identification of this first residue. Direct identification by HPLC is possible; in the protocol below the acetylamino acid is hydrolyzed with acylase, and the liberated amino acid is identified by direct amino acid analysis. Other protocols involve derivatization with phenacyl bromide and analysis of the resulting phenacylate acetylamino acids by reversed-phase HPLC (Hirano et al., 1993).

Intact protein is digested with specific endoproteases (*UNIT 11.1*) or cleaved with cyanogen bromide (*UNIT 11.4*), the newly generated amino groups are blocked, and the N-terminal Ac-peptide is unblocked with acylaminoacyl-peptide hydrolase for sequencing. The blocked N-terminal amino acid is treated with acylase and identified. In this protocol succinic anhydride is used to block newly generated peptides; in Alternate Protocol 2 phenylisothiocyanate and performic acid are used for blocking to yield phenyl carbamates.

NOTE: This protocol was developed using an LKB Alpha Plus (ninhydrin-based) Analyzer (Pharmacia Biotech) for amino acid analysis. This analyzer requires ≥0.5 to 1 nmol amino acid for a significant analysis. With more sensitive amino acid analysis methods, the amount of sample can obviously be reduced.

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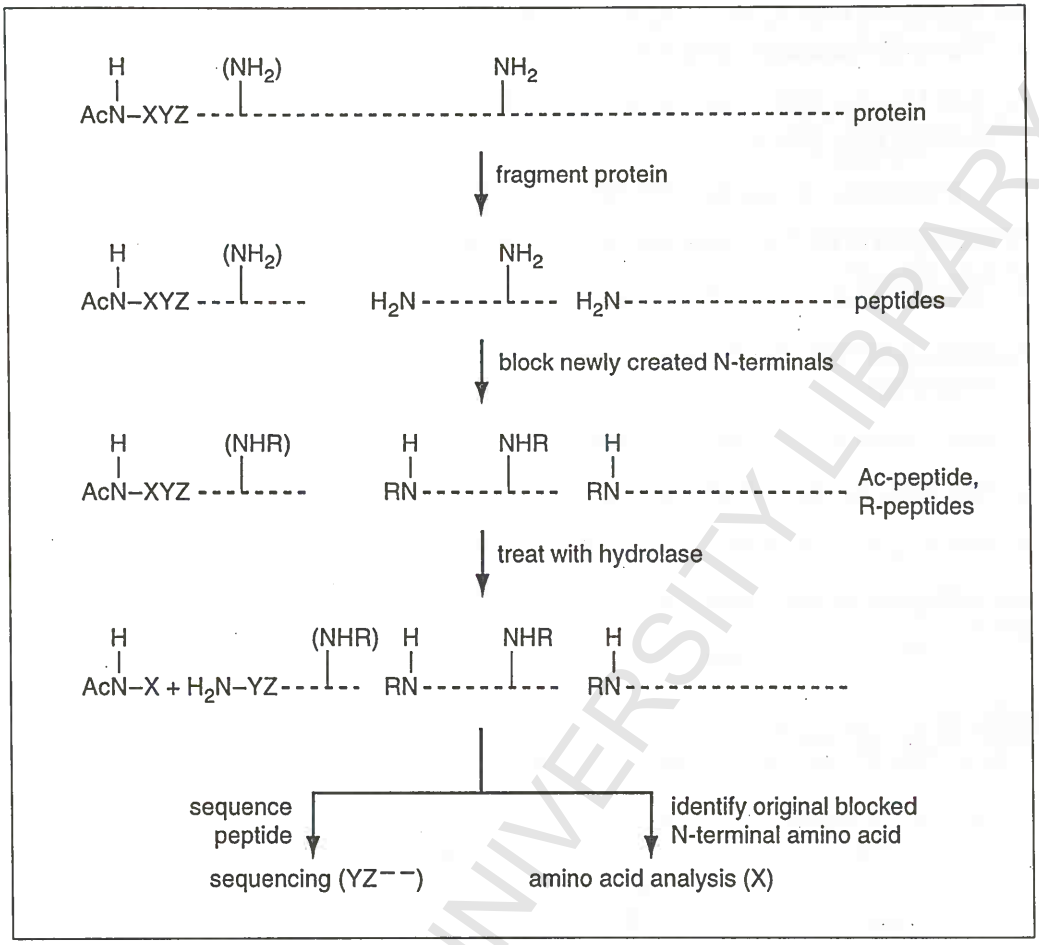

Figure 11.7.2 Strategy for the sequencing of N^{α}-acetylated proteins, unblocking with acylaminoacyl-peptide hydrolase. Symbols: Ac, acetyl group; R, amino acid side chain; X, Y, and Z, amino acid residues.

Materials

1 nmol/10 µl protein/peptide dissolved in a small volume of suitable volatile solvent
20% (v/v) acetic acid
Succinic anhydride
12% (v/v) triethylamine (TEA)
20% (v/v) trifluoroacetic acid (TFA)
Ethyl ether
Hydrolase buffer I: 50 µM sodium phosphate buffer (pH 7.2; *APPENDIX 2E*)/ 1 mM EDTA/2 mM $MgCl_2$
0.5 M NaOH
Acylaminoacyl-peptide hydrolase (EC 3.4.19.1; Pierce, Takara Biochemical, Boehringer Mannheim, or Sigma)
70% (v/v) formic acid
Acylase buffer: 100 mM sodium phosphate buffer, pH 7.0 (*APPENDIX 2E*)
Acylase I (3.5.1.14; e.g., Sigma)
0.2 M sodium citrate with pH adjusted to pH 2.2 using concentrated HCl
0.9 × 7.5–cm glass test tubes

Additional reagents and equipment for endoprotease digestion (*UNIT 11.1*) or cyanogen bromide cleavage (*UNIT 11.4*), and reversed-phase HPLC (*UNIT 11.6*)

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Generate peptides

1. Transfer 1 to 2 nmol protein in a suitable volatile solvent to a 0.9 × 7.5–cm glass test tube and lyophilize.
2. Generate peptides by digesting the protein with an endoprotease (*UNIT 11.1*) or by cleaving it with cyanogen bromide (*UNIT 11.4*).

   For endoprotease digestion, select an endoprotease with defined cleavage. Resuspend the protein in 50 µl of an appropriate buffer and add enzyme to give an enzyme/substrate ratio of 1/20 (w/w). Incubate briefly.
3. Heat the reaction mixture 5 min at 100°C or acidify it with 20% acetic acid (when carbonate buffers are used) to stop proteolysis.
4. Lyophilize the reaction products.

Succinylate α- and ε-NH_2 groups

5. Dissolve lyophilized sample in 50 µl water or carbonate buffer. Add a 200-fold molar excess of solid succinic anhydride in small portions over a span of 1 hr with vigorous mixing on a vortex stirrer. Maintain pH at 10.0 by adding 12% triethylamine.
6. Let the reaction mixture (~pH 10) stand overnight at room temperature.
7. Acidify the reaction mixture to pH 2 to 2.5 with 20% TFA. Lyophilize thoroughly.
8. Extract the residue six to ten times with 2 ml ethyl ether to remove all succinic acid and salts of triethylamine.

   The combined ether extracts can be air dried and screened for any possible presence of peptides by reversed-phase HPLC on a C18-100/300Å column (UNITS 11.6) and/or by amino acid analysis after hydrolysis.

Unblock N-terminal peptide and sequence

9. Dry residual blocked peptides.

   The residue is now invisible.
10. Add 50 µl hydrolase buffer I and adjust the pH to 7.2 with 1 to 3 µl of 0.5 M NaOH.
11. Add 2 to 5 µg acylaminoacyl-peptide hydrolase to the total peptide mixture and incubate 6 hr at 37°C.

    More enzyme can be used because the enzyme itself is an acetylated protein and hence does not give any background in sequencing. When pure enzyme is used, incubation times ≤12 hr do not give any side reactions. Although the hydrolase is quite active on short peptides, larger quantities of enzyme and longer incubation times may well be needed for longer peptides.

    Acetylated peptides with three to eight amino acids are essentially completely hydrolyzed under standard conditions unless they contain positively charged amino acids or proline; longer peptides such as the acetylated 16-residue fibrinopeptide A and the natural 13-residue Ac-α-MSH were hydrolyzed ~50% in 12 hr, but the 14-residue Ac-renin substrate was only hydrolyzed ~2% in 20 hr with 4 µg of hydrolase (Kobayashi and Smith, 1987).
12. Heat-inactivate the reaction 5 min at 100°C.
13. Lyophilize the sample.
14. Dissolve sample in 50 µl of 70% formic acid. Apply an aliquot equivalent to 0.5 nmol protein to the sequencer to identify the N-terminal sequence from the second residue onwards.

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Lysine is detected as the phenylthiohydantoin (PTH) derivative of N^{ε}-succinyl-lysine, which elutes ~1 min in front of PTH-alanine (about at the same time as N^{ε}-acetyl-lysine) in standard analysis.

Identify the blocked N-terminal amino acid

15. After removing the required aliquot for sequencing, lyophilize the remaining 0.5 to 1.5 nmol protein.
16. Dissolve lyophilized sample in 50 µl acylase buffer. Add 5 U acylase I at pH 7.0 and digest 6 hr at 37°C.
17. Lyophilize the reaction mixture.
18. Dissolve the residue in 50 µl citrate buffer, pH 2.2. Apply entire sample to an amino acid analyzer.

ALTERNATE PROTOCOL 2

REMOVAL OF ACYLAMINO ACIDS BY HYDROLASE TREATMENT USING PHENYLISOTHIOCYANATE/PERFORMIC ACID AS BLOCKING REAGENTS

This protocol is the basis for a commercially available kit for unblocking proteins (Tsunasawa et al., 1990; Hirano et al., 1993). The catalog/Technical Guide 1 from Takara Biochemical describes kits and methods for chemical and enzymatic removal of blocking groups and sequencing of N-terminally blocked protein. As indicated in Figure 11.7.2, the general strategy in this protocol is essentially the same as that in Basic Protocol 2. The two protocols differ in the chemistry for blocking newly generated peptides.

Additional Materials *(also see Basic Protocol 2)*

12.5% (v/v) trimethylamine
Phenylisothiocyanate
Benzene
Ethyl acetate
Performic acid, freshly prepared by mixing 95 µl of 99% formic acid and 5 µl of 30% hydrogen peroxide
Hydrolase buffer II: 10 mM sodium phosphate buffer (pH 7.2; *APPENDIX 2E*)/ 0.1 mM dithiothreitol (DTT)
0.5% (v/v) trifluoroacetic acid (TFA)

0.9 × 7.5–cm glass test tubes
IEC CL clinical centrifuge or equivalent

Additional reagents and equipment for endoprotease digestion (*UNIT 11.1*) or cyanogen bromide cleavage (*UNIT 11.4*), and reversed-phase HPLC (*UNIT 11.6*)

Generate peptides

1. Prepare sample and generate peptides by enzymatic or chemical cleavage, and lyophilize the reactions products as described in Basic Protocol 2, steps 1 to 4.

Block α- and ε-NH_2 groups

2. Dissolve lyophilized peptides in 100 µl of 12.5% trimethylamine. Add 10 µl phenylisothiocyanate and mix well. Incubate 1 hr at 50°C.
3. Add 250 µl benzene and mix by vortexing. Centrifuge 5 min at maximum speed in an IEC CL centrifuge (5000 rpm). Remove the upper layer. Extract the lower phase two more times with benzene.
4. Similarly, extract the remaining lower phase three times with 250 µl ethyl acetate.

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5. Lyophilize the final lower phase.
6. Repeat steps 2 to 5 once.
7. Dissolve lyophilized sample in 50 µl performic acid and mix well. Incubate 1 hr at 0°C, to convert the thiocarbamate to stable phenyl carbamate.
8. Remove the reagent under reduced pressure in a vacuum desiccator. Dissolve the residue in 100 µl cold water and lyophilize repeatedly to ensure complete removal of organic solvents.

Unblock N-terminal peptide

9. Dissolve lyophilized blocked peptides in 50 µl hydrolase buffer II. Add 2 to 5 µg acylaminoacyl-peptide hydrolase to the total peptide mixture. Incubate 6 hr at 37°C.

   The lyophilized peptides are not visible in the tube. If there is any difficulty in dissolving the substrate, sonicate the sample.

   The authors recommend 0.05 to 1.0 U of commercial acylaminoacyl-peptide hydrolase per nmol of substrate in their kit.

10. Heat inactivate the mixture 5 min at 100°C.
11. Lyophilize the reaction mixture.

Sequence the peptide

12. Dissolve reaction products in 50 µl of 0.5% TFA (or other suitable solvent). An appropriate aliquot may now be applied to the glass-fiber disc of a sequencer.

REMOVAL OF ACETYL GROUPS BY ACID HYDROLYSIS

BASIC PROTOCOL 3

The protein is digested with endoprotease (see Table 11.1.3) to release peptides with a minimum of acid-labile peptide bonds. The preferred method for preparing peptides is to digest the protein with the protease from *Staphylococcus aureus* strain V8 (endoproteinase Glu-C), which cleaves peptide bonds at the carboxyl side of aspartate and glutamate in phosphate buffer, pH 7.8 (*UNIT 11.1*). With this cleavage, the aspartyl bond (generally the most labile peptide bond) is at the C-terminus, and, at worst, acid treatment will give a high background of aspartate in the first cycle only, with relatively little interference in subsequent cycles. Peptide purification using either ion-exchange chromatography (*UNIT 8.2*) or high-performance liquid chromatography (HPLC) can be quite tedious because of the complex assay for the blocked N-terminal peptide—the blocked peptide is identified after complete enzymatic digestion with pronase and treatment of the resulting acylamino acid with acylase to release free amino acid (Chin and Wold, 1985, 1986). Because of the anticipated low yield of pure N-terminal peptide, the amount of starting protein required is about five times greater than the amount of peptide analyzed. Blocked peptides are hydrolyzed with HCl, lyophilized, and sequenced.

Materials

2 to 5 nmol blocked peptide
1 N HCl
70% (v/v) formic acid or other suitable solvent

1-ml glass vial
14-oz. propane torch
110°C oven

1. Transfer 2 to 5 nmol blocked peptide to a 1-ml vial and lyophilize.

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2. Add 200 to 500 µl of 1 N HCl and seal the vial using a 14-oz. propane torch. Incubate 10 min in 110°C oven.

   The incubation time may have to be adjusted for each individual peptide. The yield of unblocked N-terminus increases with time, but background from peptide bond cleavage also increases to complicate sequence determination. Based on the results with a limited number of proteins, 10 to 20 min appears to be optimal.

3. Immediately cool the vial on ice. Break the seal and lyophilize the hydrolysate.

4. Dissolve sample in 40 to 100 µl of 70% formic acid. An appropriate aliquot (e.g., 10 to 20 µl, or 0.5 to 1.0 nmol peptide) can now be applied to the sequencer.

   As a solvent, 70% formic acid has been found to be uniformly useful, especially for small amounts of material of low solubility in water. Additional exposure to acid does not appear to affect results. Also, 0.5% (v/v) trifluoroacetic acid (TFA) is a good solvent.

   Because unblocking is likely to be incomplete, the amount of material subjected to sequencing is increased.

ALTERNATE PROTOCOL 3

REMOVAL OF ACETYL GROUPS FROM N-TERMINAL SER AND THR WITH ANHYDROUS TRIFLUOROACETIC ACID

Treatment of proteins containing N-terminal Ac-Ser or Ac-Thr with anhydrous trifluoroacetic acid causes an N→O acyl shift and β-elimination of the acetyl esters. This procedure is adapted from Wellner et al., 1990.

Materials

Polybrene solution: 100 mg/ml Polybrene in 6.7 mg/ml NaCl (0.115 M final)
0.1 nmol/µl protein or peptide in a suitable volatile solvent
Anhydrous trifluoroacetic acid (TFA)

12-mm-diameter glass-fiber filter disc, treated with TFA (standard sequencer disc, Applied Biosystems)
1.5-ml polypropylene microcentrifuge tube with cap
45° and 65°C oven

1. Place a 12-mm glass-fiber filter disc in a 1.5-ml polypropylene microcentrifuge tube. Wet disc with 30 µl polybrene solution and dry.

2. Apply 10 to 20 µl protein or peptide solution to the filter disc and dry again.

3. Add 30 µl anhydrous TFA to the thoroughly dried filter. Close tube and incubate 4 min at 45°C.

4. Open the tube in a fume hood and let stand ~5 min to allow most of the TFA to evaporate.

5. Incubate open tube 10 min at 45°C. Close tube and incubate 16 hr at 65°C *or* 3 days at 45°C. The filter can now be applied to the pad of a sequencer.

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REMOVAL OF N-TERMINAL FORMYL GROUPS AND UNBLOCKING OF PYRROLIDONE CARBOXYL GROUPS WITH ANHYDROUS HYDRAZINE

Protein blocked at the N-terminus is incubated in the presence of anhydrous hydrazine to remove formyl groups and open the cyclic imide of pyrrolidone carboxylate; it is then used for sequencing. In treatment with hydrazine, asparagine and glutamine are converted to the corresponding hydrazides and arginine is converted to ornithine, so three additional PTH standards are required to identify these amino acids in the sequence. This procedure is taken from Miyatake et al., 1993.

Materials

~100 pmol protein, free or bound to polyvinylidine difluoride (PVDF) membrane
3-phenyl-2-thiohydantoin (PTH) derivatives of β-hydrazidyl-aspartate, γ-hydrazidyl-glutamate, and ornithine (standards)
Anhydrous hydrazine

4 × 44–mm (small) test tubes
13 × 100–mm (large) test tubes

1. Place ~100 pmol protein in a 4 × 44–mm test tube and dry.

   The sample may be protein on a membrane removed from a sequencer after obtaining evidence that the sample is blocked.

2. Place small test tube in a 13 × 100–mm test tube that contains 100 µl anhydrous hydrazine. Seal the large test tube under a vacuum.

3. Incubate the sample 8 hr at –5°C *or* 4 hr at 20°C.

   Treatment at –5°C is sufficient to remove formyl blocking groups, and treatment at 20°C is sufficient to open the cyclic imide of pyrrolidone carboxylate. Under the latter conditions, removal of acetyl blocking groups appears to be very low (<10%). At the lowest temperature, side reactions are minimal; at 20°C, only the most labile peptide bonds may be cleaved, but the side-chain amides of asparagine and glutamine are converted to the corresponding hydrazides, and arginine is converted to ornithine. The two conditions can be used in succession if the first one fails. It should be noted that the PTH derivatives of the two hydrazides elute well after leucine in a standard HPLC program; they may not be observed unless the elution time is extended.

4. Open the tube and dry 16 hr in a vacuum desiccator. The treated sample may now be sequenced.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

NED solution

Dissolve 25 mg *N*-(naphthyl)-ethylenediamine·2 HCl (Sigma; 2 mM final) in 50 ml absolute ethanol. Prepare fresh daily.

PGA digestion buffer

0.1 M $NaHPO_4$, pH 8.0
5 mM dithiothreitol (DTT)
10 mM EDTA
5% (v/v) glycerol
Cool to 4°C

Prepare fresh before use.

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PGA-N substrate

Dissolve 5.5 mg L-pyroglutamyl-β-naphthylamide (Sigma; 0.02 M final) in 1 ml methanol. Prepare fresh daily.

COMMENTARY

Background Information

Of the approximately 200 covalent modifications known to exist in proteins, the sixteen shown in Table 11.7.1 involve the N-terminus (Krishna and Wold, 1993), and all but one of these result in "blocked" N-termini that cannot be sequenced by regular Edman sequencing. The exception is acylation of the N-terminus with another amino acid, leaving a free N-terminus in which the original N-terminal amino acid has become the second residue in the sequence. Blocked N-termini have represented a major hurdle to investigators who wish to establish the N-terminal sequence of a protein. A good deal of effort has been expended in establishing methods that can be used to undo the blocking reaction and make the protein or peptide susceptible to Edman sequencing. Methods for unblocking include both chemical and enzymatic reactions. The choice of method depends on the nature of both the blocking group and the blocked N-terminal amino acid. In these protocols only the two most common blocking structures, pyrrolidone carboxyl and acetylaminoacyl terminals, are considered in detail; others (listed in Table 11.7.1) are mentioned briefly.

Enzymatic methods for unblocking N-termini are more specific than chemical methods. When it was first established that all prokaryotic proteins started with an initial N^{α}-formyl-Met, the search for the enzyme(s) responsible for the removal of the formyl (or the formyl-Met) group was immediately started, and a formyl-hydrolase was found. The occurrence of proteins with an N-terminal formyl group still attached is rare, and the reaction catalyzed by this enzyme has not become a part of the standard sequencing procedures. When it was subsequently found that a large number of eukaryotic proteins contained acylated N-termini, a similar search for acyl-hydrolases was initiated. In spite of much work in this area, no such enzyme has yet been found. Considering that initiation of protein synthesis in eukaryotes involves free Met and that any acylated derivative is thus made co- or post-translationally, presumably for a specific purpose, the absence of the sought-after unblocking hydrolases is perhaps not surprising. Similarly, there is no enzyme known to catalyze the hydrolysis (deacylation) of the cyclic amide of pyrrolidone carboxylate to convert this N-terminal blocking group to glutamate. However, there are

Table 11.7.1 N-terminal derivatives in proteins[a]

Derivative	No. carbon atoms	Reference
N^{α}-formyl	C_1	Capecchi (1966)
N^{α}-acetyl	C_2	Arfin and Bradsaw (1988)
N^{α}-acyl	C_2, C_4, C_6, C_8, C_{10}	Neubert et al. (1992)
N^{α}-myristoyl	C_{14}	Carr et al. (1982)
N^{α}-lauroyl	C_{12}	Moscarello et al. (1992)
N^{α}-tetradeca(mono and di)enoyl	$C_{14:1}$, $C_{14:2}$	Moscarello et al. (1992)
N^{α}-pyrolidone carboxyl	—	Orlowski and Meister (1971)
N^{α}-aminoacyl	—	Kaji (1976)
N^{α}-αketoacyl	—	Recsei and Snell (1984)
N^{α}-glucuronyl	—	Kolattukudy (1984)
N^{α}-glycosyl	—	Kennedy and Baynes (1984)
N^{α}-methyl	—	Siegel (1988)

[a]Taken from Krishna and Wold (1993).

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enzymes that will remove the entire blocked amino acid. One of these, pyroglutamate aminopeptidase (5-oxoprolyl peptidase; Orlowski and Meister, 1971), catalyzes removal of pyrrolidone carboxylate (pyroglutamate) from the N-terminus of proteins and peptides, and, as illustrated in the protocols in this unit, is being used very effectively to unblock proteins for sequencing. Another type of enzyme, acylaminoacyl-peptide hydrolase, catalyzes the removal of other acylamino acids from the N-terminus. In both cases, the acylated N-termini are unblocked by removing the entire N-terminal acylamino acid, leaving residue 2 as the new free N-terminus.

Pyroglutamate is found at the amino terminus of a number of naturally occurring proteins. In some cases the cyclized residue has been found to be essential for biological activity, but in other cases, such as in immunoglobulin chains, formation of pyrocarboxylic acid (PCA) does not appear to affect function (Abraham and Podell, 1981). Pyroglutamate can also form by cyclization of glutamine or glutamic acid during protein purification and sequencing. This cyclization is more likely to occur under acid conditions (Crimmins et al., 1988). The presence of PCA precludes amino-terminal sequence analysis by the widely used Edman degradation method.

The enzyme pyroglutamate aminopeptidase is present in a wide variety of plant and animal tissues and in numerous species of bacteria (Szewczuk and Kwiatkowska, 1970; McDonald and Barrett, 1986). Although the first preparations used for deblocking peptides prior to sequencing were isolated from bacteria (Doolittle and Armentrout, 1968), the present commercially available enzymes are of mammalian origin.

The enzyme acylaminoacyl-peptide hydrolase (see Basic Protocol 2 and Alternate Protocol 2) is most active with short peptides containing acetylated alanine, methionine, glycine, serine, or threonine as the N-terminus. It will also remove formyl-, propionyl-, and butyryl derivatives of these amino acids, albeit at a slower rate than for the acetyl derivatives (Gade and Brown, 1987). It is not known whether longer-chain alkanoyl derivatives are also acceptable as substrates; as more proteins are found with N-terminal saturated and unsaturated fatty acyl groups, the suitability of these derivatives as substrates for the hydrolase becomes increasingly relevant. In addition to peptide length, the nature of the amino acids in the vicinity of the N-terminus also affects enzyme activity (Krishna and Wold, 1992; Sokolik et al., 1994). An immobilized derivative of the enzyme has been prepared, making it more stable and reuseable (Farries et al., 1991). As in the case of the chemical methods, definition of a specific set of unblocking and sequencing conditions may have to be modified empirically to give optimal information yield for a particular protein.

Many of the methods for unblocking proteins use simple chemical reagents; these methods have evolved to take advantage of the unique chemical properties of each derivative. Thus, specific unblocking of acetyl-serine or acetyl-threonine can be effected through a putative β-elimination after N-O-acyl shift in anhydrous acid (Wellner et al., 1990), pyruvoyl can be converted to alanine by reductive amination (Huynh et al., 1984), and formyl and pyrrolidone carboxylate groups can be removed by mild treatment with acid or hydrazine under conditions where acetyl and longer-chain acyl groups are not cleaved (Miyatake et al., 1993). It is possible to achieve preferential cleavage of acetyl blocking groups as well (see Basic Protocol 3 and Alternate Protocol 3). The main difficulty with chemical unblocking methods is that, in the typical protein, there will always be some particularly labile peptide bonds that may be cleaved by the treatment; in fact, the various acid treatments that have been used to remove N-terminal acetate are typically equal to or harsher than those used to specifically cleave aspartate peptide bonds (Schultz, 1967; Landon, 1977). Because of this, most of the chemical methods are more reliable and specific if they can be applied to short blocked peptides, selected to be void of aspartate if possible, rather than to the intact parent proteins. This in turn means that potentially complex protein fragmentation and peptide isolation steps (*UNITS 11.1-11.6*) must be added to the general unblocking procedure.

A number of strategies have been explored to produce pure blocked N-terminal peptides. The most obvious one is to digest the protein with trypsin followed by carboxypeptidase B to remove all C-terminal lysines and arginines in the resulting peptides. In this reaction mixture, all the peptides except the N-terminal one will have the positive charge of the N-terminus; thus, these charged peptides can be retarded on a cation-exchange column, and the N-terminal blocked peptide can be found in the unretarded fraction (Chin and Wold, 1986).

Because of all the variable and unique properties of individual proteins, it is necessary to

empirically develop an approach suitable to each protein. The protocols given in this unit are based on a limited number of cases, but they should provide useful starting points for development of appropriate reactions for a particular protein.

Critical Parameters

Removal of pyrrolidone carboxylic acid

Pyroglutamate aminopeptidase activities isolated from all sources are thiol exopeptidases. The enzyme requires the presence of thiols for activation, so dithiothreitol (DTT) is included in the digestion buffer. The enzyme is inhibited by thiol-binding reagents such as iodoacetate, chloromercuribenzoate, Hg^{2+}, Cu^{2+}, Co^{2+}, and Zn^{2+} (Szewczuk and Kwiatkowska, 1970). The pH optimum of the enzyme is ~8.0.

Pyroglutamate aminopeptidase is sensitive to denaturing agents. Urea may be used at concentrations ≤1 M, but 0.5 M guanidine hydrochloride decreases enzyme activity to 79% of control values, and the enzyme is essentially inactivated at higher concentrations (Boehringer-Mannheim product information).

Enzyme specificity

Pyroglutamate aminopeptidase is specific for pyrrolidone carboxylic acid (PCA) residues at the amino terminus of peptides and proteins. The rate of removal of PCA is dependent on the adjacent residue and the enzyme does not cleave PCA-proline (McDonald and Barrett, 1986).

Highly purified pyroglutamate aminopeptidase should remove only the amino-terminal PCA residue; however, cleavage after an internal proline has been reported (Nagasawa et al., 1988). Early preparations often contained contaminating endoproteases which produced unwanted internal cleavages. When using such preparations, it was essential to repurify the desired intact protein after digestion. The protein-sequencing grade of pyroglutamate aminopeptidase presently available from Boehringer Mannheim is subjected to a quality control test designed to detect contaminating proteases. Although this should eliminate the problem, the prudent investigator will continue to be alert for evidence of additional cleavages.

Removal of N^{α}-acetylamino acids

It is well established that persistent buffering capacity in the sample to be sequenced seriously jeopardizes the effectiveness of sequencing cycles. For this reason, one of the main concerns in the various unblocking procedures is ensuring that they do not leave an excessive amount of buffers to interfere with sequencing. The two major trouble-makers in these protocols are the succinate used in blocking peptides (Basic Protocol 2), and the phosphate used in enzymatic unblocking (Basic Protocol 2 and Alternate Protocol 2). Once aware of these potential problems, it is relatively simple to develop methods to avoid them. Ether extraction and repeated lyophilization are recommended after succinylation and appear to be sufficient to eliminate interfering succinate from the sample. Using small volumes and low concentrations of phosphate buffer in enzymatic unblocking renders interference from phosphate insignificant. It is important to be aware of these potential hazards from introduced impurities to ensure that a successfully unblocked protein or peptide yields meaningful sequence information.

Troubleshooting

To the extent that unknown proteins really are unknowns, most of the protocols are strictly empirical and essentially constitute a series of troubleshooting steps. The proposed sequential exploration of hydrazine-labile, acid-labile, and enzyme-susceptible N-termini (Hirano et al., 1993), for example, is designed specifically as a progressive hunting expedition to identify the N-terminus and get sequence information beyond it. Similarly, for unknown blocked proteins, selection of appropriate unblocking procedures, choice of proper enzyme to cause fragmentation, and identification of conditions for the unblocking reaction must be made on a trial-and-error basis. When the amount of protein is limited, this is obviously not an attractive approach. For proteins whose mRNA sequence has been determined, and for which the experiments are designed to explore the extent of N-terminal processing of a given predicted structure, selection of the proper methods can be much more direct.

Pyroglutamate digestion is generally used after a sequencing attempt on a protein or peptide fails to yield sequence information, suggesting that the amino terminus is blocked. The protein is then digested with pyroglutamate aminopeptidase and a second sequencing attempt is made. A second failure to obtain amino-terminal sequence data may indicate either that the blocking group is not pyroglutamate or that enzymatic digestion has not been successful. Strategies for removing other

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blocking groups are discussed below. Several controls to demonstrate enzyme activity of the pyroglutamate aminopeptidase may be employed. Pyroglutamate aminopeptidase activity is fairly labile and sensitive to various inhibitors. Activity of the enzyme may be tested by the colorimetric method described in the Support Protocol. An internal control using a commercially available PCA-containing peptide such as bombesin may be added to the reaction. After digestion, the mixture is separated chromatographically and the peptide subjected to sequence analysis. Crimmins et al. (1988) and Dimaline and Reeve (1983) describe digestion of bombesin and other peptides and separation of unblocked from blocked forms.

If the enzyme is active in the protein mixture, variations in enzyme/substrate ratio and digestion times should be investigated. The rate of cleavage of PCA is dependent on the nature of the adjacent residue. If the rate is low, increasing digestion time or temperature may be necessary. Additional aliquots of enzyme may be added at intervals during digestion to compensate for enzyme lability.

If the substrate protein is large with substantial tertiary structure, the amino terminus may be inaccessible to the enzyme. The protein should be reduced and alkylated before digestion with pyroglutamate aminopeptidase. Denaturing the protein by making a concentrated solution in 8 M urea or 6 M guanidine·HCl and then diluting to ≤1 M urea or ≤0.5 M guanidine·HCl may be helpful in exposing the amino-terminus. It may be necessary to cleave the protein with an endoprotease and isolate the amino-terminal peptide before digestion with pyroglutamate aminopeptidase. Digestion of small peptides tends to occur more easily than digestion of large proteins.

If more than one residue is exposed at the amino terminus, it may indicate that the enzyme preparation contains additional endoprotease activities. A purer preparation of enzyme may alleviate this problem. Alternatively, the digestion products may be separated chromatographically before sequence analysis. Two amino-terminal residues have also been detected when the residue adjacent to the original PCA is a glutamine (Crimmins et al., 1988). Presumably some of this second glutamine cyclized to PCA, which was then removed by the enzyme.

Anticipated Results

With Basic Protocol 1, 200 pmol protein should yield 40 to 100 pmol unblocked protein ready for sequence analysis. Digestion of electroblotted proteins (Alternate Protocol 1) is somewhat less efficient but fewer sample-handling steps are required. Depending on the sensitivity of the protein sequencer, 50 pmol starting protein should produce a detectable signal of 10 to 20 pmol.

When combining treatment with hydrolase and proteolysis (Basic Protocol 2 and Alternate Protocol 2), the results can be quite variable because the activity of the hydrolase depends on peptide structure. In a test of eight known acetylated proteins, 500 pmol starting material produced sequences as short as two residues and as long as twelve residues. Under optimized conditions for acid hydrolysis (Basic Protocol 3), 40% to 60% of total peptide yields unblocked N-terminal residues. Treatment with trifluoroacetic acid (Alternate Protocol 3) has given yields of 3% to 40% unblocked N-terminals. Treatment with hydrazine (Alternate Protocol 4) has yielded 50% to 80% unblocking of formylated proteins and 69% to 90% of pyrrolidone-carboxylated proteins.

Time Considerations

For Basic Protocol 1 with a reduced and alkylated protein sample that can be placed directly into PGA digestion buffer, setting up the digestion takes ~30 min and should be started early in the morning so the first digestion step, which requires 7 to 9 hr, can be carried out during the remainder of the working day; the second digestion is carried out overnight. Dialysis requires 3 to 4 hr; alternatively, reversed-phase desalting combined with isolation of the blocked protein can generally be accomplished in 60 to 90 min by reversed-phase HPLC. In Alternate Protocol 1, which starts with a blotted protein, preparation and preliminary incubation requires ~90 min and digestion requires 23 hr. Support Protocol 1 takes ~3.5 hr to complete.

Treatment with hydrolase combined with proteolysis (Basic Protocol 2 and Alternate Protocol 2) requires 1 to 2 days depending on the method for blocking newly generated amino groups. Acid hydrolysis (Basic Protocol 3) can be completed in 0.5 day. Treatment with trifluoroacetic acid (Alternate Protocol 3) requires 2 to 4 days, and treatment with hydrazine (Alternate Protocol 4) takes 1.5 days.

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C-Terminal Sequence Analysis

Carboxy-terminal (C-terminal) sequence analysis is used for direct confirmation of the C-terminal sequence of native and expressed proteins, for detection and characterization of protein processing at the C-terminus, for identification of post-translational proteolytic cleavages, and for obtaining partial sequence information on N-terminally blocked protein samples in order to facilitate design of oligonucleotide probes for gene cloning. Although recent advancements and the availability of an automated chemical C-terminal sequencing methodology are rapidly making this approach the preferred method for obtaining C-terminal sequence information, it may still be desirable for some investigators to use alternative sequencing methods—e.g., when automated instrumentation is not available in the laboratory and when verifying and confirming automated sequencing data by an additional different technique is desired. This unit describes an automated chemical method (see Basic Protocol 1) and a manual enzymatic method (see Basic Protocol 2) for determining C-terminal sequence information. Carboxypeptidase digestion (Basic Protocol 2) requires only a standard amino acid analysis method (*UNIT 3.2*).

AUTOMATED C-TERMINAL SEQUENCE ANALYSIS

BASIC PROTOCOL 1

Until recently, practical implementation of automated C-terminal sequencing in most laboratories has been hindered by limitations of the technology (see Background Information). The HP G1009A C-terminal protein sequencing system (Hewlett-Packard) represents the first commercially available instrument for automated C-terminal sequence analysis. This instrument was originally introduced in 1994 with the capability of generating one- to three-amino-acid-residue sequence assignments for any of the twenty commonly occurring amino acids using nanomolar amounts of sample. Recent advances have extended the number of callable cycles from three to five and have permitted "high-sensitivity" C-terminal sequence analysis of samples <100 pmol (Miller and Bailey, 1994). This has resulted in the general applicability of C-terminal sequencing to samples purified by SDS-PAGE.

Automated C-Terminal Sequencing Instrumentation

The HP G1009A C-terminal sequencing system from Hewlett-Packard (see *SUPPLIERS APPENDIX*) consists of an HP G1000A C-terminal protein sequencer, an HP1090M liquid chromatograph, an HP Vectra 486/66 MHz computer with Microsoft MS-DOS and Windows environment, HP specialty C-terminal sequencing reagents and solvents, HPLC columns and solvents, and all related consumables. The G1009A C-terminal sequencing system is operated and maintained as recommended by the manufacturer.

Sample Preparation

Application of liquid samples to Zitex membranes. Protein samples for C-terminal sequencing are applied directly to 1 × 12–mm Zitex (porous Teflon; Hewlett-Packard) membranes that have been prewet with isopropanol. The sample solution (50 pmol in 1 to 10 µl volume) is applied directly to the wetted membrane, and the membrane is allowed to dry 10 to 20 min at room temperature. When the sample volume is >10 µl, repeated additions of 10-µl aliquots are performed; the entire sample should be added before the membrane is completely dried. Alternatively, the Zitex strip can be wet with isopropanol and inserted, while still wet, into ≤3 ml solution containing 150 to 200 pmol of the protein of interest. The solution is stirred overnight at room temperature, then the membrane is removed using forceps and allowed to air dry. Once dry, the membrane is inserted into a C-terminal sequencer column (an inert Kel-F column fitted with inert endfrits) and installed in any one of four sample positions on the HP G1009A sequencer.

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Preparation of samples blotted from SDS-PAGE gels. Experimental procedures have been developed for the C-terminal sequence analysis of proteins purified by SDS-PAGE (Burkhart et al., 1995; Miller and Bailey, 1995). Typically, 50-pmol (1- to 10-μg) samples are loaded onto individual SDS-polyacrylamide gel lanes and electrophoresed (*UNIT 10.2*). Following the electrophoretic resolution of the protein, the protein bands on the SDS gel are electroblotted to a Zitex membrane (*UNIT 10.7*; Burkhart et al., 1995; Miller and Bailey, 1995). Proteins are visualized by staining with sulforhodamine b (Coull and Pappin, 1990), then 2 × 12–mm strips containing protein bands are cut from the Zitex membrane and inserted directly into the C-terminal sequencer columns as either single or multiple bands.

Sequencing Chemistry

Sequencer column reactions that occur on the Zitex membrane include chemical coupling with diphenyl phosphoroisothiocyanatidate (DPP-ITC), cyclization of the C-terminal residue to a thiohydantoin, and cleavage to release the thiohydantoin amino acid derivative (see Fig. 11.8.1). The thiohydantoin amino acid is then extracted from the Zitex membrane in the sequencer column to the sequencer transfer flask, where it is prepared for HPLC injection. Chemical coupling is performed by first reacting the sample with DPP-ITC in ethyl acetate/heptane, then washing it with organic solvent (a mixture of ethyl acetate and acetonitrile) to remove excess DPP-ITC. The intermediate peptidylphosphoryl anhydride is reacted with a solution of pyridine in ethyl acetate to effect a five-membered ring closure to the carboxy-terminal peptidylthiohydantoin. The chemical coupling reaction is repeated two more times to ensure complete conversion of the C-terminal amino acid to a thiohydantoin. After washing with organic solvent, the peptidylthiohydantoin is then chemically cleaved with potassium trimethylsilanolate in alcoholic solvent (methanol and *t*-butanol) to generate a shortened peptide, ready for continued rounds of sequencing, and a thiohydantoin amino acid. The thiohydantoin amino acid is extracted to the injection flask with ethyl acetate/acetonitrile mixture, dried, and redissolved for injection onto the online reversed-phase HPLC. The thiohydantoin amino acid is identified and quantitated by comparison to the retention times of defined quantities of the online thiohydantoin amino acid standards.

HPLC Analysis of Thiohydantoin Amino Acid Derivatives

The G1009A C-terminal sequencing system provides automated HPLC analysis of sequencer cycles using the HP 1090M liquid chromatograph with filter photometric detection at 269 nm and the 2.1-mm × 25-cm HP specialty reversed-phase phenylthiohydantoin (PTH) analytical C18 HPLC column. A 35-min binary gradient using the ion-pairing reagent alkyl sulfonate yields highly reproducible elution times and peak resolution. The gradient consists of a 4-min isocratic elution with solvent A (phosphate buffer, pH 2.9) followed by a continuous gradient to solvent B (acetonitrile/water) at a flow rate of 0.2 ml/min at 40°C. A stable thiohydantoin amino acid standard mixture is incorporated on the sequencer for online automated peak calibration and quantitation; the standard mixture consists of the synthetic thiohydantoin derivatives corresponding to the actual sequencing products for each of the twenty amino acids.

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MANUAL C-TERMINAL SEQUENCING USING CARBOXYPEPTIDASES

BASIC PROTOCOL 2

Many different methods to provide C-terminal sequence information have been described. One of the more common techniques involves digestion of the purified, intact protein or peptide with one or more carboxypeptidases, which are exopeptidases that remove amino acids, one at a time, from the C-terminus of a protein. After digestion, released amino acids are separated from the protein and analyzed by amino acid analysis (*UNIT 3.2*). Once the kinetics of the reaction for the individual amino acids present at the C-terminus are determined by a preliminary experiment, a quantitative digestion is performed. The relative amounts of amino acids released over time are used to obtain C-terminal sequence information for the protein in question.

Carboxypeptidases have a wide range of specificity for individual amino acids and therefore cleave different amino acid residues at very different rates. Carboxypeptidase B predominantly cleaves Lys and Arg residues. Carboxypeptidases A, P, and Y cleave most amino acids, although Carboxypeptidase A will not cleave Pro or Arg residues. In addition, cleavage by Carboxypeptidase A is greatly slowed by Gly residues; Carboxypeptidases P and Y are also greatly retarded by Ser and Asp, respectively. Combinations of enzymes can be used individually and mixed together to provide further sequence information and to help with amino acid assignments (see Anticipated Results and see Fig. 11.8.6).

The following procedure assumes that an unlimited quantity of protein is available. It can also be performed for some proteins with as little as 300 pmol of protein (Michel et al., 1993). This protocol describes a reaction using carboxypeptidases P and Y. Other carboxypeptidases may be used in the same way, provided the appropriate buffer is used (see supplier's information).

Materials

Purified protein
Digestion buffer: 100 mM *N*-ethylmorpholine, adjusted to pH 6.0 with acetic acid (NEM, Aldrich; store at 4°C)
Carboxypeptidases A, B, P, and/or Y (sequencing grade, Boehringer Mannheim)
Aminobutyric acid (Sigma)
Trifluoroacetic acid (TFA, Fluka)
Titertubes with plugs (Bio-Rad)
5-kDa-MWCO and (optionally) 30- and 10-kDa-MWCO Ultrafree filtration devices (Millipore)
Additonal reagents and equipment for SDS-PAGE (*UNIT 10.1*) and amino acid analysis (*UNIT 3.2*)

Prepare sample and controls

1. Dissolve 4 mg (~40 nmol) purified protein to a concentration of 1 mg/ml in digestion buffer. Heat 3 min at 100°C to denature the protein.

   The protein concentration should be as high as possible.

   Using 100 mM N-ethylmorpholine, pH 6.0, as the digestion buffer provides good activity for carboxypeptidases P and Y.

2. Add 400 ng (~4 nmol) aminobutyric acid to the 4 ml protein solution to monitor recovery. Add 100 ng (~1 nmol) aminobutyric acid to 1 ml of digestion buffer.

   Aminobutyric acid is used as an internal standard, as it is not usually found in proteins. Norleucine may also be used (see Critical Parameters).

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3. Add 1 ml protein solution to each of three reaction Titertubes labeled 1, 2, and 3. Add 200 µl protein solution to each of three control Titertubes labeled 4, 5, and 6.

Titertubes are preferred because they tend to give high recoveries with low amounts of protein.

Prepare the enzymes

4. Dissolve one vial each of carboxypeptidases P and Y in separate 50-µl aliquots of water.

5. Heat 10 µl of each enzyme 3 min at 100°C to inactivate the enzyme.

Digest the protein

6. Add 20 µl carboxypeptidase P to tubes 1 and 2. Add 20 µl carboxypeptidase Y to tubes 2 and 3. Add 5 µl inactivated carboxypeptidase P to tubes 4 and 5. Add 5 µl inactivated carboxypeptidase Y to tubes 5 and 6. Incubate at room temperature.

7. Remove 200-µl aliquots from reaction tubes 1, 2, and 3 at 1, 10, 60, and 240 min. Remove 200-µl aliquots from control tubes 4, 5, and 6 at 240 min only.

8. Stop the reaction by adding 22 µl TFA to each aliquot as it is removed.

Analyze released amino acids

9. Pass each aliquot through a 5-kDa-MWCO Ultrafree filtration device to separate released amino acid residues from the shortened peptide and the carboxypeptidases.

A combination of 30-kDa-, 10-kDa-, and 5-kDa-MWCO filters may be used to separate large proteins from the enzyme(s) and amino acids (see Critical Parameters).

10. Wash the filter with 1 vol digestion buffer.

11. Collect the filtrates and use either rotary evaporation or lyophilization to dry them completely.

12. *Optional:* Wash the retentate with digestion buffer or other suitable buffer and retain this sample, which contains protein and carboxypeptidases, for future analysis by SDS-PAGE (*UNIT 10.1*) or comparative peptide mapping (see Troubleshooting). Store at –20°C.

13. Perform amino acid analysis on all samples and controls. For each amino acid, plot amount recovered versus time of digestion (see Fig. 11.8.6). From the preliminary experimental data, choose a time for quantitative digestion that produces >50% of expected yield for at least one amino acid and intermediate times that enable discrimination of rates of release.

If rates of release are extremely slow, the ratio of enzyme to substrate or the incubation temperature can be increased. Alternatively, the reaction can be slowed by decreasing the temperature or the amount of enzyme.

COMMENTARY

Background Information

Automated methods

Several automated methods for C-terminal sequence analysis of peptides and proteins have been described during the last few years (for recent reviews see Inglis, 1991, and Bailey, 1995). Although all have shown promise, most relied on chemistry that was incapable of derivatizing one or more of the 20 naturally occurring amino acids (Inglis et al., 1991; Bailey et al., 1992a; Boyd et al., 1992), and all involved the use of specialized instrumentation and/or reagents that were not commercially available and hence not easily obtainable by the protein sequencing community (Inglis et al., 1991; Bailey et al., 1992b, 1993; Boyd et al., 1992). These limitations have prevented the

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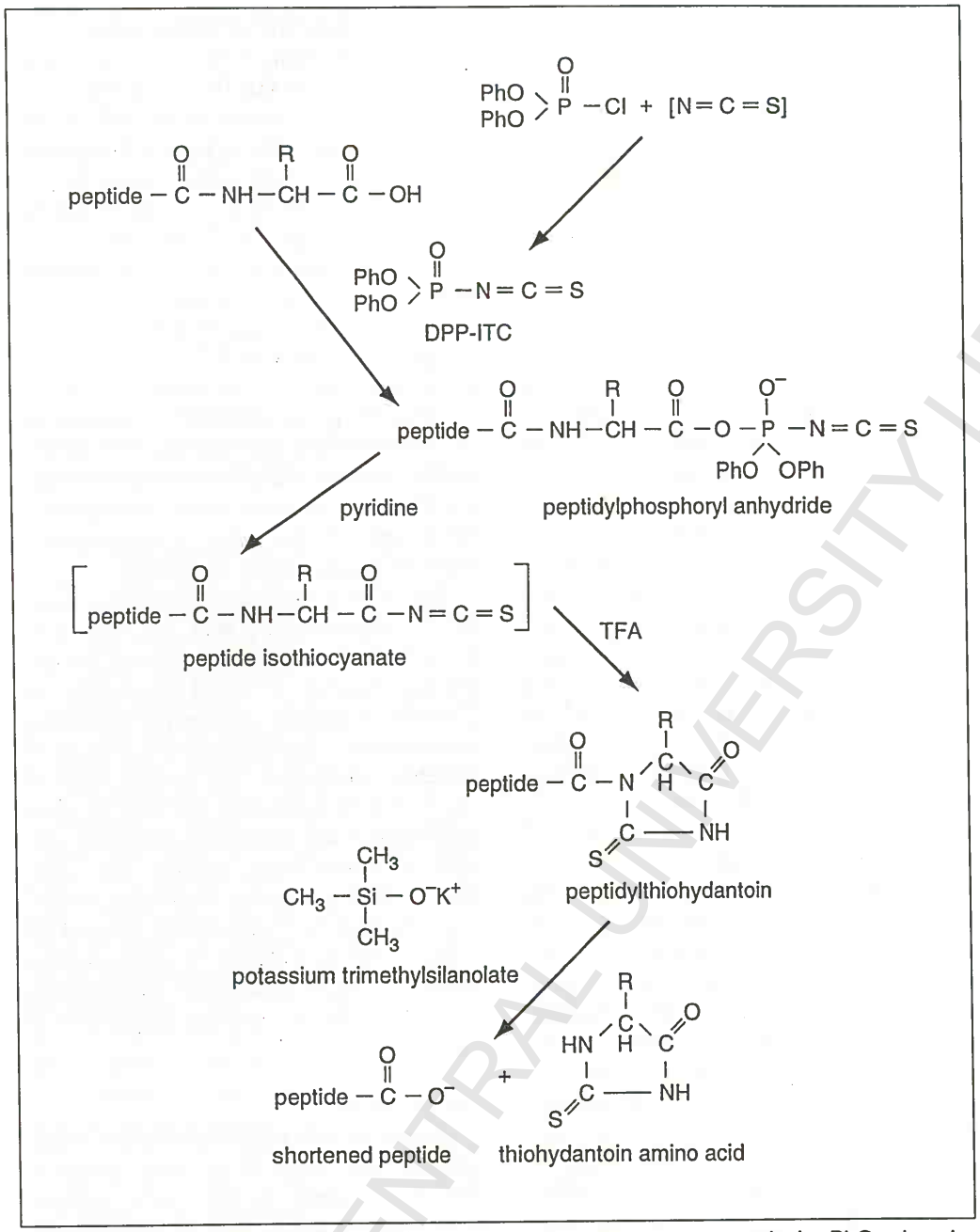

Figure 11.8.1 Chemical scheme for automated C-terminal sequence analysis. PhO, phospho group.

practical implementation of automated C-terminal sequencing technology in most laboratories.

Recently, these limitations have been mitigated by both the advent of a chemistry for C-terminal sequence analysis that is capable of derivatizing all of the 20 common amino acids (Bailey et al., 1992a, 1995) and the introduction of an instrument based on this chemistry by Hewlett-Packard (Miller and Bailey, 1994).

The overall chemical reaction scheme for C-terminal sequencing is depicted in Figure 11.8.1 and consists of two principal reaction events. The α-carboxylic acid group of the C-terminal amino acid residue of the protein is modified to a chemical species that differentiates it from the other constituent amino acid residues in the protein sample. This modification involves chemically coupling the C-terminal amino acid residue with the sequencing coupling reagent diphenyl phosphoroisothiocyanatidate (DPP-ITC). The modified C-terminal amino acid residue is in a chemical form that permits it to be easily cleaved from the rest of the protein molecule. The cleavage reaction generates the uniquely modified C-terminal

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amino acid residue as a thiohydantoin amino acid derivative (TH-AA), which is detected and identified using HPLC analysis. The remaining component of the cleavage reaction is the protein shortened by one amino acid residue; the shortened protein is subjected to the next round of the coupling/cleavage cycle. Thus the overall sequencing process is a sequential chemical degradation of the protein that yields successive amino acid residues from the C-terminal; the released amino acid residues are detected and identified by HPLC.

As is shown in Figure 11.8.1, the coupling reaction of the sequencing cycle begins with activation of the carboxylic acid function of the C-terminal amino acid by treatment with trifluoroacetic acid (TFA); this acid treatment promotes the reaction with the coupling reagent, DPP-ITC. The reaction is accelerated in the presence of pyridine; this suggests the formation of a peptidyl phosphoryl anhydride species as a plausible reaction intermediate. The final product of the coupling reaction is the peptidylthiohydantoin, which is formed by chemical cyclization of the peptide isothiocyanate intermediate. The peptidylthiohydantoin is then treated with TFA in order to stabilize peptidylthiohydantoin formed from C-terminal proline (Bailey et al., 1995). This acid treatment has no effect on peptidylthiohydantoins formed from the other 19 amino acids. The peptidylthiohydantoin, which bears the chemically modified C-terminal amino acid residue, is cleaved with potassium trimethylsilanolate (a strong nucleophilic base) to release the C-terminal thiohydantoin amino acid derivative for subsequent analysis and the shortened peptide for the next cycle of chemical sequencing. The thiohydantoin derivative of the C-terminal amino acid residue is detected chromatographically and identified by HPLC analysis with detection at a UV wavelength of 269 nm.

Because the thiohydantoin amino acids produced have UV absorption spectra and extinction coefficients similar to those of the phenylthiohydantoin amino acids formed during Edman degradation (Cromwell and Stark, 1969), the sensitivity of C-terminal sequencing is expected to rapidly approach that currently possible with N-terminal sequencing.

Because the sequencing products formed from serine and cysteine yield the same degradation product (dehydroalanine thiohydantoin), the residue assignment of cysteine in unknown sequences requires prior chemical modification of cysteine (S-alkylation; see *UNIT 15.1*), as is routinely done in amino-terminal sequencing. Similarly to the phenylthiohydantoin (PTH)-Ile from N-terminal sequencing, the thiohydantoin (TH)-Ile analog elutes as a doublet due to formation of the allo isomer during synthesis of thiohydantoin. This results in the formation of two diastereomeric derivatives of TH-Ile, which are resolved during the separation (see Figs. 11.8.2 and 11.8.5).

The thiohydantoin amino acid standard HPLC chromatogram (e.g., see Figs. 11.8.2 and 11.8.5) shows the elution times for each of the 20 common amino acid derivatives (identified by single-letter abbreviations; see Table A.1A.1). The peak labeled S/C corresponds to the dehydroalanine thiohydantoin formed during the sequence analysis of serine and cysteine. The peaks labeled D′ and E′ correspond to the B- and γ-methyl esters of thiohydantoin aspartate and glutamate, respectively.

The chemical method is universal for any of the 20 common amino acids and easily produces the thiohydantoin derivatives of Ser and Thr that frequently are found to constitute the C-termini of proteins. Although the method is currently able to provide three to five cycles of sequence information, it is the first cycle of sequence information that provides the most unambiguous and definitive information, because all protein forms—whether they result from internal processing, unspecified proteolytic cleavages or fragmentations induced by isolation and purification, or single-residue truncations during purification—are available for analysis in their relative proportions at cycle 1.

Manual methods

Manual methods of determining C-terminal sequence have relied on enzymatic digestion with carboxypeptidases followed by analysis of the released amino acids by conventional amino acid analysis. Carboxypeptidases are exopeptidases that remove amino acids one at a time from the C-terminus of the protein. Ideally amino acids are released in order of their position from the C-terminus; thus the C-terminal sequence is deduced from the rate of appearance of released amino acids. In practice the rates of release are strongly influenced by the nature of the side-chains of both the terminal and penultimate amino acids, as well as by the general protein structure. Thus the kinetics of release are highly dependent on the nature of the particular protein and in the most unfavorable situations will not yield interpretable sequence data. Recent methods using mass spectrometry to examine the "ladder" of par-

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Table 11.8.1 Relative Rates of Release of Amino Acids by Carboxypeptidases

Carboxy-peptidase	Release rate[a]				References
	Rapid	Slow	Very slow[b]	None[b]	
A	Y,F,W,L,I,M,T, Q,H,A,V,Hsr	N,S,K,$MetSO_2$	G,D,E,$CysSO_3H$, CMCys	P,R,Hypro	Ambler, 1972
B	K,R			all except K,R, penultimate P	Ambler, 1972
P	R,A,N,E,Q,H,I,L, K,M,F,P,W,Y,V	D,T	S,G	penultimate P,G	Hofmann, 1976; Lu et al., 1988
Y	Y,F,W,L,I,M,V	P,A,E,S,T,Q, N,CMCys	D,H,R,K,G	penultimate G	Jones, 1986

[a]Amino acids are indicated using single-letter abbreviations (see Table A.1A.1). Modified amino acids: CMCys, carboxymethyl cysteine; $CysSO_3$, cysteic acid; Hsr, homoserine; Hypro, hydroxyproline; $MetSO_2$, methionine sulfone.

[b]The presence of one of these amino acids in the penultimate position generally decreases the rate of release of the terminal amino acid.

tially digested peptides in the reaction mixture (Rosnack and Stroh, 1992; Patterson et al., 1995) offer a means of identifying the C-terminal sequence that avoids the problems inherent in following digestion kinetics.

Carboxypeptidases have a wide range of specificity for individual amino acids and therefore cleave different amino acid residues at very different rates. Carboxypeptidase B predominantly cleaves Lys and Arg residues. Carboxypeptidases A, P, and Y cleave most amino acids, but the rates of cleavage are reduced significantly by the presence of Gly residues. Carboxypeptidase A will not cleave Pro or Arg residues. Cleavage rates for carboxypeptidases P and Y are also greatly retarded by Ser and Asp, respectively. Combinations of enzymes can be used individually and mixed together to provide further sequence information and to help with amino acid assignments. Table 11.8.1 summarizes information on the rates of release of particular amino acids by various carboxypeptidases.

The four carboxypeptidases described above are available commercially; the procedures for their use are straightforward and rapid. The digestion products may be analyzed by amino acid analysis or mass spectrometry of the truncated peptides (see Chapter 16). Thus manual sequencing using enzymatic digestion offers a relatively low-cost method of obtaining C-terminal sequence information without the need for a dedicated instrument.

Critical Parameters

Automated methods

Samples for C-terminal sequencing are prepared by a single-step application of the protein or peptide to a Zitex membrane. Polyvinylidene difluoride (PVDF) membrane should not be used, because it is unstable under the basic conditions used during the cleavage reaction. No presequencing attachment (covalent coupling) chemistries are required. All samples for C-terminal sequence analysis are noncovalently absorbed to the Zitex supports. Elimination of the covalent coupling requirements common to earlier methods (Inglis et al., 1991; Bailey et al., 1992a; Boyd et al., 1992) permits all of a particular sample to be available for C-terminal analysis. This sample application method is amenable to product formulations, as well as samples that are isolated using routine separation procedures involving various buffer systems, salts, and detergents. The matrix components are conveniently eliminated during the initial steps of the sequencing cycle. Additionally, no presequencing chemical modifications or derivatizations are required other than routine cysteine alkylation if cysteine identification is desired, thus avoiding any sequencing ambiguities associated with incomplete or nonspecific amino acid side-chain reactions.

There are no special "Begin" cycles: all cycles are identical. Additionally, there are no modifications to the cycle program required to sequence blotted samples versus directly applied liquid samples.

Manual methods

Enzymes and specificities. Several sequencing-grade carboxypeptidases are available from commercial vendors. The substrate specificities are diverse enough that at least one enzyme or a combination of enzymes should be able to remove amino acids from the C-ter-

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minus of almost any protein. Mixtures of two or more carboxypeptidase enzymes can initiate further digestion that can provide insight into the sequence of a protein.

An enzyme/substrate ratio of 1/100 is usually sufficient; however, altering the ratio is one easy way of manipulating the reaction rate (Ambler, 1972). Most carboxypeptidases have significant catalytic activity at 25° as well as 37°C (Lu et al., 1988), and the reaction temperature can also be modified to change the reaction rate as desired. Stopping the reaction can easily be accomplished by adding enough trifluoroacetic acid (TFA) to the reaction mixture to bring the pH to 2.5 to 3.0 (adding TFA to 10% of final volume is usually sufficient to inactivate the enzyme; Lu et al., 1988).

Buffer system. Due to the sensitivity of amino acid analysis to buffer salts, it is wise to use volatile buffers for buffering the digestion reaction. *N*-ethylmorpholine (NEM) is commonly used with acetic acid to provide a volatile buffering system compatible with most carboxypeptidases. A 200 mM NEM/acetic acid buffer is usually sufficient, but it is best to follow the enzyme supplier's recommendations for ionic strength and pH conditions.

Denaturing protein. Unless the C-terminus is known to be accessible in the protein's native state, denaturation is usually recommended. Product literature from enzyme suppliers lists the compatibility of denaturants with enzymatic activity. Most laboratories should be familiar enough with the behavior of the protein of interest to arrive at a suitable denaturation scheme. Disulfide bridges are usually not problematic during exopeptidase digestion.

Time course. The classic limitation of carboxypeptidases is that the rate of release is highly dependent on the amino acid side chains of both the terminal and penultimate amino acids. For this reason, it is highly recommended that a preliminary experiment be done to understand the kinetics of the reaction for a particular protein. Suggested time points for a preliminary experiment are 0, 1, and 5 hr. These should provide enough information to choose time points for the quantitative experiment; the selected time points should enable discrimination of the rates of release of several amino acids and provide a final yield of amino acid that is a substantial portion of the input substrate (i.e., >50%). In general, the reaction is sampled more frequently during the initial stages. Digestion for up to 16 to 24 hr may be required.

Preparing samples for amino acid analysis. Previous investigators have used precipitation with trichloroacetic acid (TCA) or sulfosalicylic acid to separate the protein from the released amino acids (Mondino et al., 1972). Alternatively, disposable filtration units are available with different membranes that provide different specific molecular weight cutoffs. Ultrafree units (Millipore) are designed to filter ~350 µl in a standard microcentrifuge.

Depending on the size of the protein under analysis, one round of filtration through a 5-kDa-MWCO filter, followed by a single washing step, can very effectively separate amino acids from protein. If the protein under scrutiny is particularly large, it may be necessary to filter the sample first through a wide-pore (e.g., 30-kDa) filter and then through a 5-kDa filter. If the protein of interest is significantly different in molecular weight from the carboxypeptidase used, then sequential filtration can also potentially yield relatively pure digested protein sample free of carboxypeptidase contamination. Carboxypeptidase Y, for example, has a molecular weight of 61 kDa. By passing the mixture of carboxypeptidase Y (61 kDa), low-molecular-weight protein (e.g., 25 kDa), and amino acids (<0.2 kDa) first through a 30-kDa filter and then through a 5-kDa filter, each component can be purified.

Controls. It is particularly important to run inactivated enzyme controls, because sequencing-grade enzymes contain salts and possibly impurities that create artifacts which are most easily identified by comparing the controls to the samples.

Internal standards are essential for interpreting the amino acid analysis data from these experiments. An internal standard is added to the initial reaction mixture at an amount usually equal to 10% of the theoretical amount of each amino acid to be released; this places the standard in the range of the released amino acids (~10% of the maximum theoretical yield is usually recovered). Aminobutyric acid and norleucine are suitable amino acid standards that are not usually found in proteins.

Troubleshooting

Automated methods

For troubleshooting assistance for automated C-terminal sequence analysis contact Hewlett-Packard Technical Support (see *SUPPLIERS APPENDIX*).

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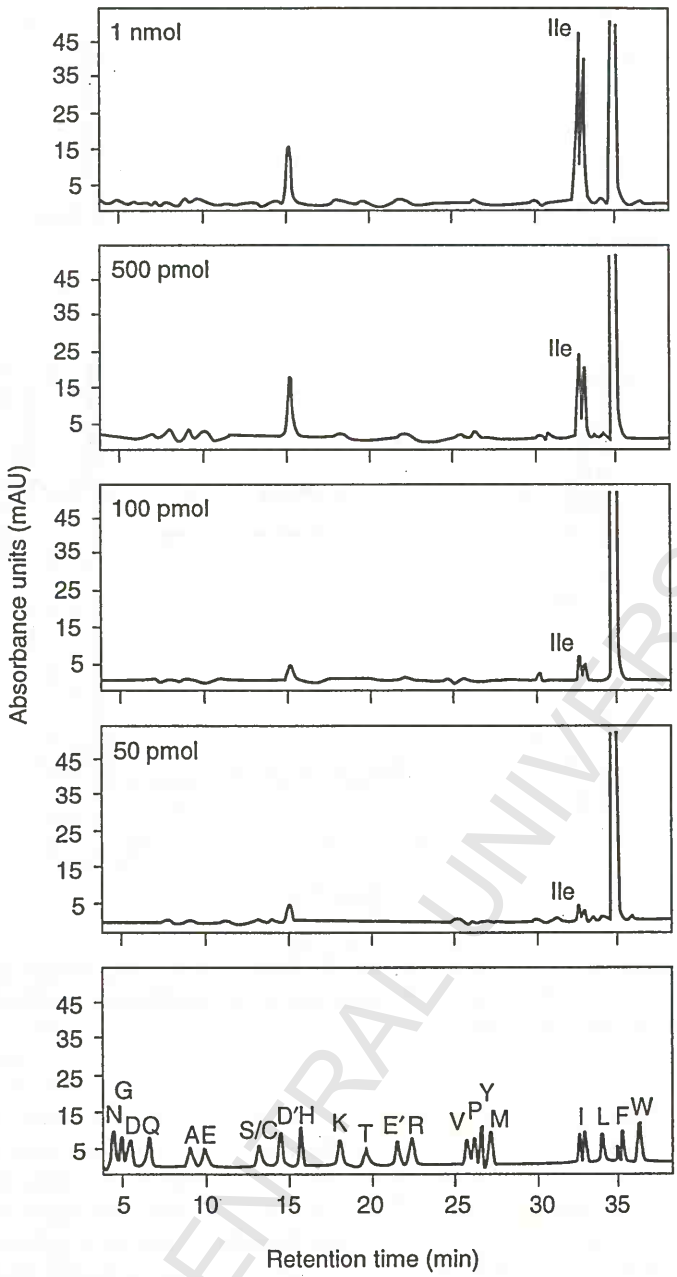

Figure 11.8.2 C-terminal sequence analysis of 1 nmol, 500 pmol, 100 pmol, and 50 pmol bovine β-lactoglobulin A. The cycle 1 Ile is unambiguously assigned for each sample amount. The 100-pmol thiohydantoin amino acid standard is shown in the bottom panel.

Manual methods

If no amino acids are detected by the preliminary experiment, the first suggestion is to perform a new digestion with a different enzyme or combination of enzymes. If a combination of enzymes is used, the active pH ranges for the enzymes must overlap. Most often this second experiment will provide insight into the initial failure.

If the digested protein from the experiment was not discarded, it can be analyzed by comparative peptide mapping (Isobe et al., 1986). Peptide maps on both the intact and digested proteins can usually be used to infer which peptide or peptides in the chromatogram is the C-terminal peptide, based on the loss or decrease in one or more peak and the appearance of one or several other peaks. This peptide can, of course, be collected and analyzed further.

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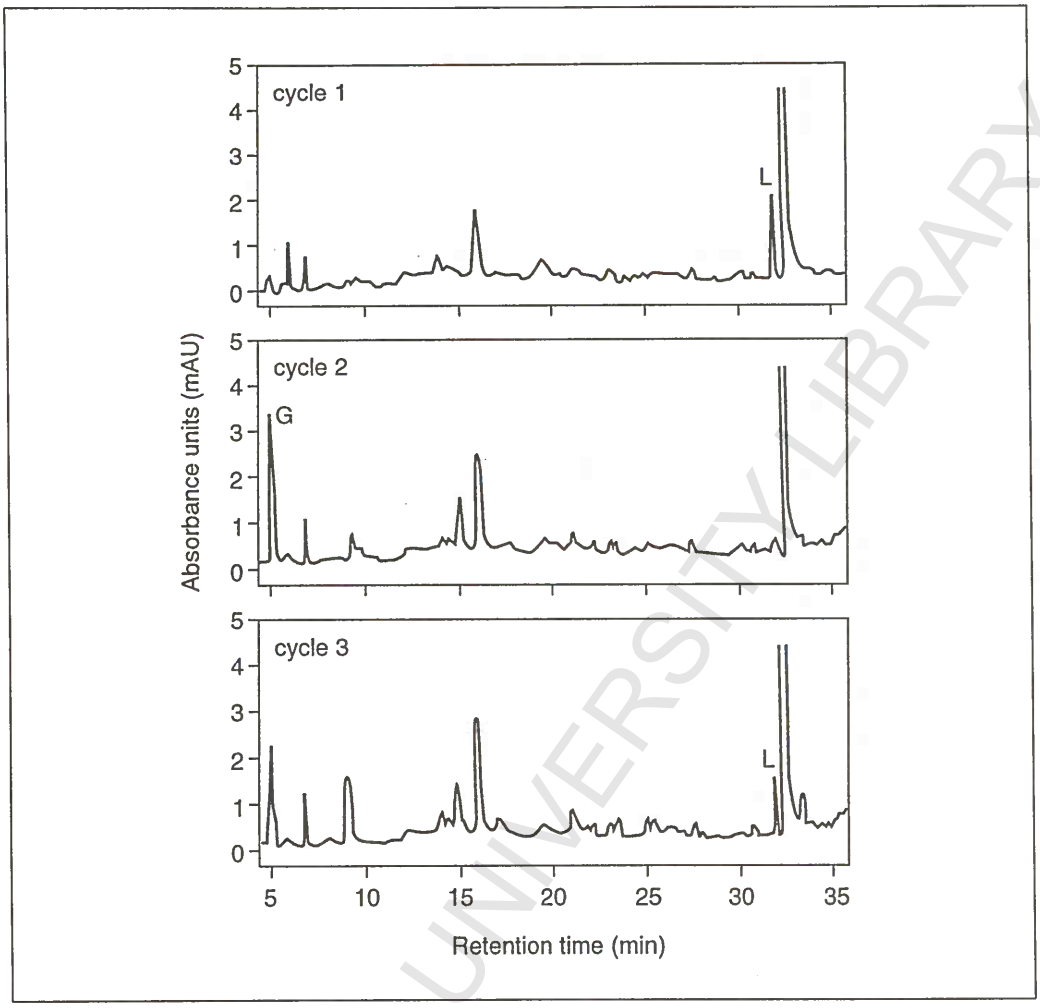

Figure 11.8.3 Cycles 1 to 3 of C-terminal sequence analysis of 50 pmol human serum albumin applied to a Zitex membrane. The sequence assignments are identified as Leu(1)-Gly(2)-Leu(3). In cycle 1, 28 pmol Leu was recovered.

An additional method for C-terminus identification is the use of a phenylenediisothiocyanate (DITC) membrane or resin in combination with Endo-Lys C digestion. The DITC moiety binds to peptides with a C-terminal Lys. Shimadzu (see *SUPPLIERS APPENDIX*) has recently introduced a method that uses a resin to which the DITC moiety has been bound (Nokihara, 1992). Eluting the unbound peptide is easily accomplished, and subsequent analysis (e.g., mass spectrometry and Edman degradation) can be performed immediately.

Aside from the automated technique, the most promising method for obtaining C-terminal sequence data is the use of mass spectrometry (MS; see Chapter 16) coupled with "ladder-generating" techniques (Patterson et al., 1995), usually either carboxypeptidase digestion (see Basic Protocol 2; Rosnack and Stroh, 1992) or C-terminal chemical truncation (Takamoto et al., 1995). Recent improvements in the accuracy and feasibility of MS procedures have allowed for accurate mass determinations of mixtures of protein molecules differing by only one amino acid. There are several limitations with this type of method; one is that the accuracy of the information obtained decreases quickly with protein size, so that it is currently most promising for peptides. This technique can be performed without the use of a truncation agent to assess the level of carboxyterminal heterogeneity (Horn et al., 1995).

Anticipated Results

Automated methods

Analysis of sequencing results is done by interpretation of the HPLC chromatogram. The G1009A C-terminal sequencing system utilizes the HP Chemstation software platform for automatic data processing and report generation. Visually, the amino acid thiohydantoins

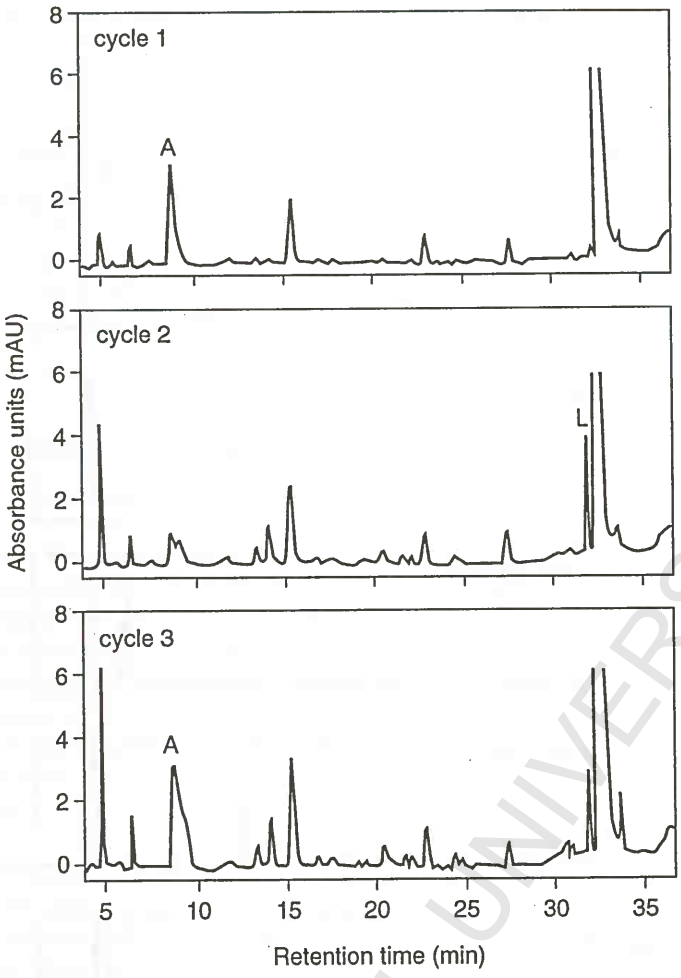

Figure 11.8.4 Cycles 1 to 3 of C-terminal sequence analysis of 125 pmol bovine serum albumin electroblotted from an SDS-polyacrylamide gel to a Zitex membrane for C-terminal sequence analysis. The sequence assignments are identified as Ala(1)-Leu(2)-Ala(3). In cycle 1, 70 pmol Ala was recovered.

can be assigned by comparing the succeeding ($n + 1$) and preceding ($n - 1$) chromatograms with respect to the cycle (n) in question. The amino acid assignments are determined by comparison of the retention times of the thiohydantoin amino acids in each cycle with the retention times of the online standard amino acid thiohydantoins (e.g., see Figs. 11.8.2 and 11.8.5).

The results of C-terminal sequence analysis of 1 nmol, 500 pmol, 100 pmol, and 50 pmol bovine β-lactoglobulin A are shown in Figure 11.8.2 in the form of the cycle 1 HPLC chromatograms obtained for each of the respective sample amounts. The recovery for the first cycle of sequencing is ~40% to 50% (based on the amounts applied to the Zitex membranes) and an approximately linear recovery is observed across the range of sample sizes. The appropriate linear response in detection of the thiohydantoin amino acid sequencing products and a sufficiently quiet chemical background permit high-sensitivity C-terminal sequence analysis.

The unambiguous residue assignments for the first three cycles of sequence analysis of human serum albumin are shown in Figure 11.8.3. In this case, a 50-pmol sample was applied to the Zitex membrane and analyzed.

Analytical gels are routinely used for analysis of protein samples and recombinant products to assess sample homogeneity. The all-too-common observation of several closely resolved bands or unexplained smaller-sized bands is frequently an indication of either cellular processing events or fragmentations in-

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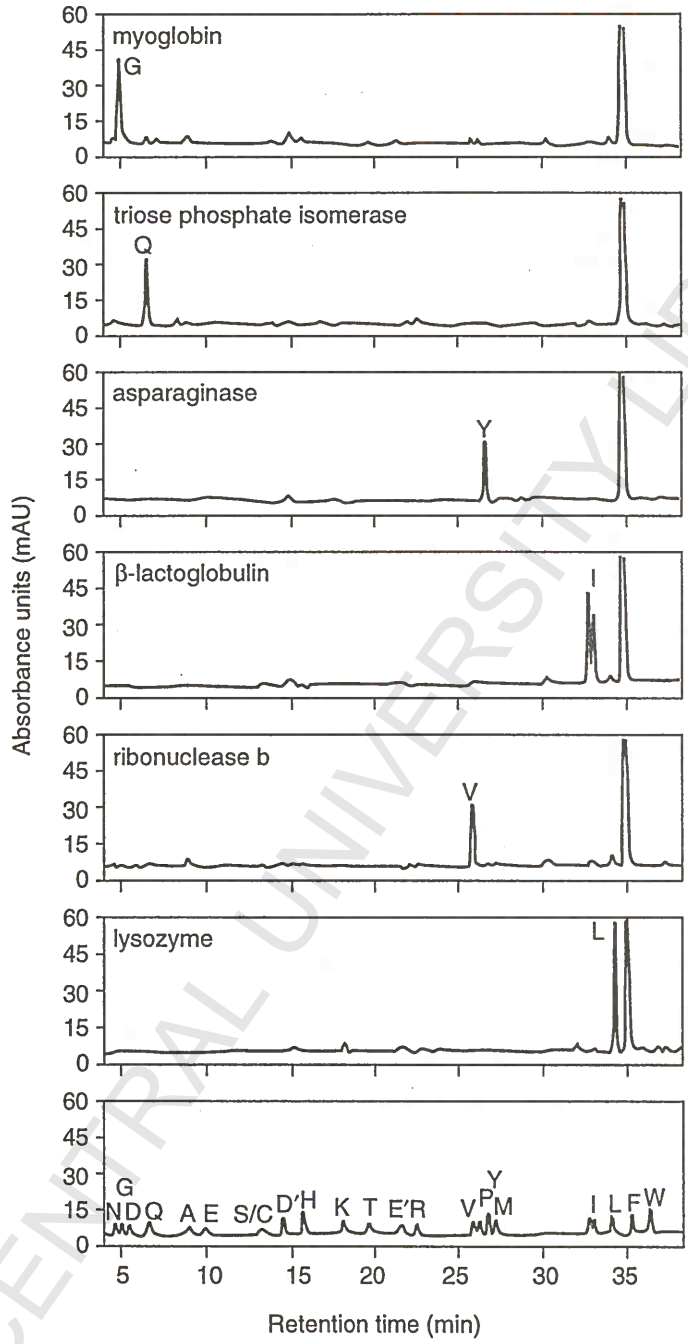

Figure 11.8.5 Cycle 1 of automated C-terminal sequence analysis of 1 nmol of a variety of proteins. A thiohydantoin amino acid standard is shown in the bottom panel.

duced by isolation and purification protocols. Automated C-terminal sequence analysis provides a direct, rapid method for characterization of these various protein species and facilitates examination of their origins and control. The result of automated C-terminal sequence analysis of a protein sample electroblotted to Zitex from an SDS-polyacrylamide gel is shown in Figure 11.8.4. Approximately 50 pmol bovine serum albumin (68 kDa) was loaded in each lane of an SDS gel, subjected to electrophoresis, and electroblotted to a Zitex membrane. Typically, a 50% efficiency of transfer from the gel to the Zitex membrane is observed. The visualization stain does not interfere with sequence analysis, and it is not necessary to treat the excised bands with any extensive wash protocol prior to C-terminal

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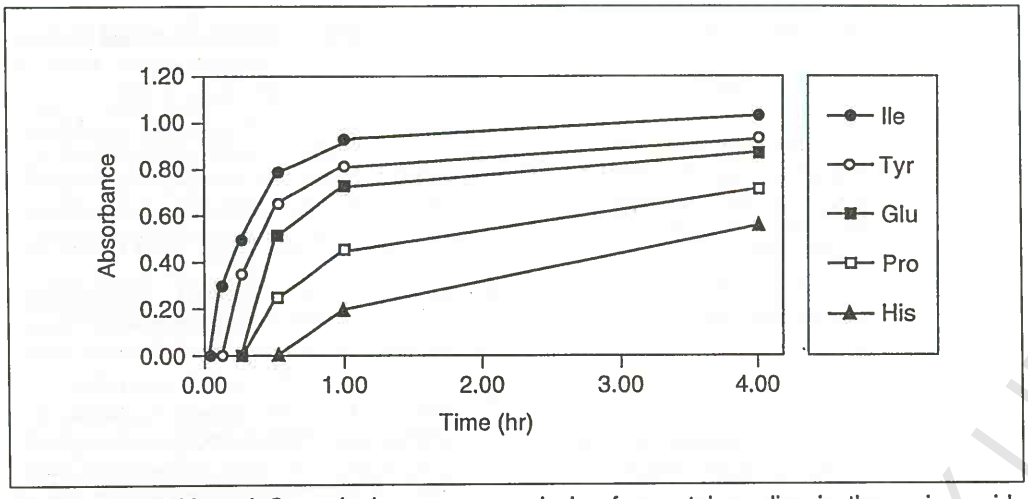

Figure 11.8.6 Manual C-terminal sequence analysis of a protein ending in the amino acids His-Pro-Glu-Tyr-Ile.

sequence analysis. The chromatograms for cycles 1 to 3 for five combined 1-cm protein bands (~125 pmol applied to the sequencer) sequenced off the Zitex electroblot demonstrate the high sensitivity and utility of the automated C-terminal sequencing technique.

Figure 11.8.5 shows cycle 1 of automated C-terminal sequence analysis of a variety of protein samples.

Manual methods

The data from manual C-terminal sequencing are usually expressed according to the following function (Hayashi, 1977):

$$[\text{AA, released}]_{\text{theoretical}} = [\text{Standard}]_{\text{theoretical}} \; [\text{Standard}]_{\text{found}} \times [\text{AA, released}]_{\text{found}}$$

and are plotted with nanomoles released on the *y* axis and time on the *x* axis (see Fig. 11.8.6).

This plot is a best-case scenario for a protein that ends in His-Pro-Glu-Tyr-Ile. In practice, the data can be more difficult to interpret. The order of the amino acids may be difficult to infer because of the different reaction rates. Also, losses of individual amino acids, presence of repetitive amino acids in a sequence, and certain other factors increase the difficulty in making specific sequence assignments using only this method. However, the method is particularly useful to confirm an automated analysis or other data suggesting the C-terminal sequence. It is also particularly useful in comparing the C-terminal sequence of preparations of the same material.

Time Considerations

For automated C-terminal sequencing, sample application to Zitex, including drying time, is generally complete within 30 min. The time for one cycle of C-terminal sequencing is ~1 hr.

Manual C-terminal sequencing, which involves significant hands-on time, can easily be performed in the course of 1 day plus overnight automated amino acid analysis HPLC of the samples.

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General description of the techniques and carboxypeptidases.

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Amino Acid Analysis

Amino acid analysis (AAA) is one of the best methods to quantify peptides and proteins. AAA also provides valuable qualitative measurement, such as quality control evaluation of synthetic peptide and recombinant protein structures, identification of proteins based on composition, and detection of odd amino acids. AAA has been used for years in primary structural studies, for example, in designing fragmentation strategies, identifying exopeptidase reaction products, and quantifying sequence samples in order to discriminate between useful data, background contamination, and N-terminally blocked samples. In addition, AAA has applications in the characterization of feed and food samples and physiological fluids.

Two general approaches to quantitative AAA exist, namely, classical postcolumn derivatization following ion-exchange chromatography and precolumn derivatization followed by reversed-phase HPLC (RP-HPLC). Excellent instrumentation and several specific methodologies are available for both approaches, and both have advantages and disadvantages as outlined in Background Information. This unit focuses on picomole-level AAA of peptides and proteins using the most popular precolumn-derivatization method, namely, phenylthiocarbamyl amino acid analysis (PTC-AAA). It is directed primarily toward those interested in establishing the technology with a modest budget. The Basic Protocol describes PTC derivatization and analysis conditions, and Support and Alternate Protocols describe additional techniques necessary or useful for most any AAA method—e.g., sample preparation, hydrolysis, instrument calibration, data interpretation, and analysis of difficult or unusual residues such as cysteine, tryptophan, phosphoamino acids, and hydroxyproline.

STRATEGIC PLANNING

Although straightforward in principle, it is far from simple in practice to achieve picomole-level quantitative accuracy in analysis of amino acids in peptides and proteins. Before embarking on amino acid analysis (AAA) bench work, strategic consideration is encouraged regarding sample amount, work load, costs, time commitment, and resource facility options (see Background Information). With a low or intermittent need for the technology, utilizing the services of an appropriate resource facility will probably be more cost-effective and expedient than performing the analysis oneself. For those who decide to establish laboratory AAA capability, the following strategic issues should be considered prior to bench work.

Most AAA methods involve the following steps: (1) peptide or protein sample preparation; (2) hydrolysis of the peptide or protein into amino acids; (3) derivatization of the amino acids for detection; (4) calibration of the HPLC system with standard amino acids and chromatographic analysis of the experimental hydrolysate; and (5) calculations and data interpretation. For postcolumn derivatization AAA, steps 3 and 4 are reversed. Various chemicals can be used for derivatizing the amino acids, but all five steps must succeed for high-quality results.

AAA is complicated by the variable susceptibility of amino acids and peptide bonds to acid hydrolysis, variable levels of background contamination, and potentially variable instrument and human performance. Overall success and useful results require meticulous work habits, reproducible instrument performance, and care at each step of the procedure.

Satisfactory instrument performance will be easier to maintain if an HPLC system is dedicated to the analysis of amino acids rather than used part-time for some other purpose.

Contributed by John W. Crabb, Karen A. West, W. Scott Dodson, and Jeffrey D. Hulmes

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Furthermore, if a significant, long-term need for the technology exists, a dedicated PTC amino acid analyzer supported by an instrument vendor is strongly recommended. The most expensive piece of equipment is the HPLC system, and the available budget will dictate HPLC options; a simple gradient HPLC system with a recording integrator will suffice; however, an automatic sample injector is also recommended.

This technology is labor-intensive. Successful AAA requires technical, mechanical, analytical, and quantitative skills, as well as common sense. Laboratory experience with general protein chemistry techniques and with HPLC instrumentation is very useful.

Sample Preparation

To obtain the most accurate PTC-AAA data, peptide and protein samples should be homogeneous (i.e., exhibit a single SDS-PAGE band and/or a single N-terminal amino acid). In addition, the sample should be dissolved in a volatile solvent, free of salts and detergents. Common volatile solvents useful for transferring readily soluble samples to hydrolysis tubes include water (HPLC grade), aqueous 0.1% trifluoroacetic acid/acetonitrile, 30% to 50% methanol, ethanol, or acetonitrile, 0.1% to 1% *N*-ethylmorpholine acetate, pH 8.5, and dilute (0.1%) HCl. Less soluble samples can be dissolved in 75% to neat organic acids such as formic, acetic, and trifluoroacetic acids. Whenever possible, enough sample should be prepared for duplicate analyses per hydrolysis (plan on ~0.5 to 1.0 μg per analysis). Useful data can be obtained with <0.5 μg of sample, but ubiquitous contamination makes routine analyses below this range more labor-intensive and difficult for any AAA procedure. Useful PTC-AAA data can be obtained from some dilute salt solutions (Dupont et al., 1989). For example, samples dried from 1 mM MOPS, pH 7 (3-[*N*-morpholino]propanesulfonic acid), or from 25 mM Tris (pH 7.2)/1 mM DTT/1 mM EDTA can yield high-quality data provided the total salt in the hydrolysis/PTC derivatization reaction is kept low (e.g., <50 μg per hydrolysis). Several specific methods for sample preparation are described: reversed-phase HPLC (see Support Protocol 1), micropreparation concentration (see Support Protocol 2), microdialysis (see Support Protocol 3), acetone preciptation (see Support Protocol 4), and ether precipitation (see Support Protocol 5).

As an aid for quantitation, a defined amount of an internal standard may be added to the sample prior to hydrolysis and/or derivatization. Subsequent data analysis is used to normalize recovery to the initial conditions. Use of internal standards in amino acid analysis is essential for some experimental applications, such as C-terminal sequencing with carboxypeptidases (*UNIT 11.8*), but in general it is optional and a matter of personal preference. Good internal standards are stable to the hydrolysis, derivatization, and analysis conditions to which they are subjected and must not coelute with any other peak in the chromatography system. Odd amino acids such as α-aminobutyric acid, norvaline, norleucine, and ornithine can be useful as internal standards in PTC-AAA.

Peptide/Protein Hydrolysis

Key to the success of most AAA is the hydrolysis of peptides and proteins into free amino acids. Complete hydrolysis is generally performed by heating the sample in constant-boiling HCl; however, variable lability of the residues complicates the process, and only sixteen of the common twenty amino acids are usually measured from HCl hydrolysates—Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, and Lys. For example, Trp and Cys are destroyed by standard HCl hydrolysis, Ser and Thr are partially destroyed, Ile and Val are slow to cleave, Met is subject to oxidation, and Asn and Gln are deamidated. Shorter hydrolysis times can be used to optimize recovery of Ser and Thr, and longer hydrolysis times may be used to optimally quantify Ile and Val. Cys must be

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modified for quantification by AAA (see Support Protocols 12 and 13). Special hydrolysis conditions must be used to measure Trp (see Support Protocols 14 and 15) and phosphoamino acids (see Support Protocols 16 and 17). Manual vapor-phase (see Support Protocol 7) and liquid-phase HCl hydrolysis (see Support Protocol 8) protocols work well and are widely used for analysis of the common residues in hydrolysates. The liquid-phase HCl hydrolysis method presented is a simple approach for hydrolyzing multiple samples of small amounts. Vapor-phase hydrolysis methods are now preferred by many analysts because they are more rapid than the liquid-phase methods, and they reduce the possibility of introducing background contaminants with HCl. Directions for cleaning hydrolysis tubes (see Support Protocol 6) are also provided. Microwave hydrolysis (see Support Protocol 10) and HCl hydrolysis on polyvinylidene difluoride (PVDF) membranes (see Support Protocol 9) are optional approaches.

Phenylisothiocyanate Derivatization

PTC-AAA typically identifies the sixteen common amino acids in peptide/protein hydrolysates, including proline. The derivatization reaction is relatively tolerant to variations in pH and molar ratios of reagents to sample, but a slow, variable degradation of the derivatives can occur in solution at room temperature. The derivatization protocol may also be successfully performed with ethanol in place of methanol, and triethylamine (TEA) substituted for *N*,*N*-diisopropylethylamine (DIEA), but resulting reagent by-product peaks will differ slightly from those shown in Figure 11.9.1 (Bidlingmeyer et al., 1984; Tarr, 1986).

The Basic Protocol describes a procedure for manual PTC derivatization. Alternatively, automatic PTC derivatization with on-line HPLC analysis can be performed with instrumentation marketed by PE Applied Biosystems. This instrumentation provides savings in time and labor as well as reduction in variability due to time-dependent degradation of the PTC derivatives. Automatic on-line vapor-phase HCl hydrolysis can also be performed with the PE Applied Biosystems model 420H hydrolyzer/derivatizer. Detailed comparisons of the performance of automatic and manual hydrolysis/PTC derivatization methods and applications of the automatic PTC-AAA technology are available elsewhere (West and Crabb, 1990, 1992).

Quantitative Chromatographic Analysis

Reversed-phase conditions for separating PTC amino acids are well established for a number of HPLC instruments with a variety of columns; HPLC vendors are usually willing to recommend and sometimes even demonstrate specific columns, solvents, and gradient conditions useful for their HPLC system. Request HPLC system information regarding optimization of the HPLC analysis of PTC amino acids from the vendor.

The PTC derivatization process normally produces UV-absorbing by-products that appear on the chromatogram (Fig. 11.9.1). These by-products, which come from reactions between PITC and other derivatization reagents and sample components, commonly include phenylthiourea (PTU, reaction with ammonia), aniline (hydrolysis product), ethylphenylthiourea (EPTU, reaction with ethylamine from DIEA), isopropylphenylthiourea (IPTU, reaction with isopropylamine from DIEA), and diisopropylphenylthiourea (DIPTU, reaction with diisopropylamine from DIEA). These chromatography peaks usually are not problematic provided they are well resolved from the amino acids. Increased amounts of by-products usually indicate that fresh derivatization reagents are needed. To minimize PTU, avoid ammonia-containing solvents, and keep ammonia vapors away from the derivatization reaction and PTC workstation.

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In addition to the column and HPLC system, important chromatography parameters for reversed-phase resolution of PTC derivatives are acetonitrile concentration, ionic strength and pH of the solvents, and temperature of the chromatography. Constant temperature is needed for reproducibility. Increasing the acetonitrile concentration and temperature causes all the PTC derivatives to elute with shorter retention times. Lowering the solvent pH increases the retention of Asp and Glu, whereas raising the solvent pH generally causes Asp and Glu to elute sooner. High-purity solvents and reagents (HPLC grade) are essential. The elution position of all the PTC amino acids in the chromatography system should be determined before attempting to calculate response factors (i.e., identify all the peaks from analysis of Pierce Standard H).

Calculations and Data Interpretation

Amino acid compositional data are commonly expressed as mole % or residues per molecule. The use of a desktop computer with spreadsheet software (e.g., Microsoft Excel) greatly facilitates amino acid analysis data reduction. Mole % (i.e., residues per 100 residues) may be used for data interpretation without knowing the identity or molecular weight of the sample.

Peptide or Protein Identification

Amino acid compositional data can be used to search computer databases using PROPSEARCH and/or ExPASy to identify unknown samples (see Support Protocol 19).

BASIC PROTOCOL

PHENYLTHIOCARBAMYL AMINO ACID ANALYSIS

Phenylthiocarbamyl amino acid analysis (PTC-AAA) is an established method for picomole-level quantitative analysis based on the reaction of phenylisothiocyanate (PITC) with amino groups to form phenylthiocarbamyl amino acid derivatives, which are then separated by reversed-phase HPLC and identified and quantified by ultraviolet absorbance. Since its introduction in the 1980s (Koop et al., 1982; Heinrikson and Meredith, 1984; Tarr, 1986), PTC-AAA has become the most popular precolumn-derivatization approach to AAA and may be used today with greater frequency than the classic ninhydrin postcolumn-derivatization method developed in the 1950s (Moore and Stein, 1949; Moore et al., 1958). During the 1990s, the majority of PTC-AAA has been performed with instrumentation from two vendors. Waters Associates first commercialized the PTC method under the trade name PicoTAG amino acid analysis (Bidlingmeyer et al., 1984, 1986), and PE Applied Biosystems Division introduced instrumentation in 1986 that performs automatic PTC derivatization with on-line HPLC analysis. The PE Applied Biosystems instrument was subsequently modified to also perform automatic on-line HCl hydrolysis. Both companies have provided reliable instrumentation and valuable technical and service support for PTC-AAA. However, most other companies marketing HPLC systems also provide instructions for how to separate PTC amino acids using their respective instrumentation. Protocols in this unit can be used with essentially any HPLC system.

Materials

Peptide or protein samples

Volatile solvents, e.g., HPLC-grade water (Burdick and Jackson); aqueous 0.1% trifluoracetic acid/acetonitrite; 30% to 50% methanol, ethanol, or acetonitrite; 0.1% to 1% *N*-ethylmorpholine acetone, pH 8.5; or 0.1% HCl.

Internal standard, e.g., α-aminobutyric acid (optional)

2:1 (v/v) methanol (Burdick and Jackson)/diisopropylethylamine (DIEA; PE Applied Biosystems)

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7:2:2 (v/v/v) methanol/DIEA/5% (w/v) phenylisothiocyanate (PITC; Pierce or PE Applied Biosystems) in heptane (Burdick and Jackson)
EDTA-containing transfer buffer compatible with HPLC system, e.g., 29 mM sodium acetate buffer pH 5.2 (Mallinkrodt), containing 0.25 mg/ml K_3EDTA (Sigma)
Amino acid Standard H (Pierce)
Peptide or protein standard, e.g., insulin subunits, myoglobin, and lysozyme (Sigma) or 7% (w/v) bovine serum albumin (National Institute for Standards and Technology, formerly National Bureau of Standards)

Vacuum system consisting of:
  Vacuum pump (e.g., Leybold-Heraeus Trivac D2A)
  Vacuum gauge (e.g., Fredericks Televac)
  Vacuum hose and connectors
  Cold trap and Dewar flask
  Glass three-way stopcock
  Polytetrafluoroethylene (PTFE) 6-mm bore Rotofol valve (Corning) or two-way stopcock
Argon (or nitrogen) gas supply consisting of:
  Tank of prepurified gas
  Regulator for gas tank
  Silicone tubing
Gradient HPLC system consisting of:
  Two HPLC pumps
  Mixing chamber
  Controller
  UV detector
  Manual injection valve
  Automatic sample injector (recommended but optional)
  Recording integrator (e.g., Shimadzu C-R6A Chromatopac)
Mininert slide valves (Pierce)
40-ml screw-cap vials (Pierce) with Teflon/silicone discs
6 × 50–mm glass Pyrex tubes (Corning)
Spreadsheet software (e.g., Microsoft Excel)

Additional reagents and equipment for preparing samples (see Support Protocols for Sample Preparation), hydrolyzing samples (see Support Protocols for Hydrolysis), performing quantitative RP-HPLC (*UNITS 8.2 & 11.6*), plotting results to determine linear response ranges, and calculating standard deviations to determine reproducibility

Set up the PTC-AAA workstation

1. Assemble key equipment and accessories close at hand in a dedicated PTC-AAA work area near a fume hood. A PTC-AAA workstation consists of a vacuum system, a fume hood, an argon (or nitrogen) flush system, an oven, freezer, HPLC system, automatic pipettor, 6 × 50–mm glass hydrolysis/derivatization tubes, 40-ml screw-cap vials, and Mininert slide valves.

Waters markets a PicoTAG Work Station (Bidlingmeyer et al., 1986) designed after the manual sequencing and PTC-AAA station developed by Tarr (1986). Original workstation descriptions with schematic diagrams appear in the above articles, and photographs of workstations are also available (Kuhn and Crabb, 1986). The work area can be arranged according to individual preference. The vacuum/argon flush system, 6 × 50–mm tubes, and 40-ml reaction vials with Mininert slide valves particularly facilitate manual acid hydrolysis and PTC derivatization protocols.

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2. Modify a few Mininert slide valves. First, remove the Teflon slider and silicone plug, enlarge the bore to ~1/8 in. (~3 mm) with an electric drill, and connect the modified valve to the vacuum system via vacuum hose and a PTFE Rotofol valve or two-way stopcock.

   This allows vacuum drying of PTC-derivatized samples in a 40-ml screw-cap vial.

3. On other Mininert valves, replace the silicone plug with the unused Teflon slider from the first modified valves, cut off the excess Teflon plug with a razor blade, push the valve open (green position in), and enlarge the bore slightly to ~3/64 in. (~1.2 mm) with an electric drill (Bidlingmeyer et al., 1986; Kuhn and Crabb, 1986; Tarr, 1986).

   This allows effective argon flushing and evacuation of samples in a 40-ml screw-cap vial in preparation for acid hydrolysis.

   CAUTION: *With extended use, the Teflon liner of the valve compresses and may no longer seal properly. Discard and replace worn valves as necessary—e.g., when the indentation in the valve becomes significant or the vacuum no longer holds.*

4. Modify several 40-ml screw-cap vials. Have a glass blower form a bulge around the circumference of the vial ~50 mm from the bottom.

   This prevents HCl condensate from running down the inside wall of the vial into the 6 × 50–mm sample tubes during acid hydrolysis.

5. Connect the vacuum and argon flush systems via a three-way stopcock (Tarr, 1986) to provide alternating gas flush and vacuum to samples in preparation for hydrolysis. Attach a short piece of vacuum hose to the third port on the three-way stopcock and notch the other end to fit snugly around the slider knobs on the Mininert valve.

Prepare the sample

6. Prepare the peptide or protein sample (see Support Protocols for Sample Preparation) and dissolve in a volatile solvent.

   Whenever possible, prepare enough sample for duplicate analyses per hydrolysis (~0.5 to 1.0 μg sample is needed per analysis; additional sample material is necessary to quantify unusual amino acids). Best results are obtained with homogeneous samples, free of salts and detergents. Common volatile solvents for readily soluble samples are HPLC-grade water; aqueous 0.1% trifluoroacetic acid/acetonitrile; 30% to 50% methanol, ethanol, or acetonitrile; 0.1% to 1% N-ethylmorpholine acetate, pH 8.5; and 0.1% HCl. Less soluble samples may require 75% to 100% formic, acetic, or trifluoroacetic acids. Samples dried from 1 mM MOPS, pH 7, or from 25 mM Tris·HCl, pH 7.5/1 mM DTT/1 mM EDTA may yield satisfactory data, if the total salt is <50 μg.

   A defined amount of an internal standard (e.g., α-aminobutyric acid) may be added to the sample prior to hydrolysis and/or derivatization; indeed, an internal standard is essential for C-terminal sequencing with carboxypeptidases (UNIT 11.8). The internal standard should be stable to hydrolysis, derivatization, and analysis conditions and should be clearly resolved on the HPLC system.

Hydrolyze the sample

7. Hydrolyze the sample (see Support Protocols for Hydrolysis).

   Hydrolysis, a key step in determining success in PTC-AAA, is usually performed by heating samples in scrupulously clean hydrolysis tubes (see Support Protocol 6), in constant-boiling HCl (see Support Protocols 7 to 9). An alternate method is microwave hydrolysis (see Support Protocol 10). Automatic on-line vapor-phase HCl hydrolysis can also be done with the PE Applied Biosystems model 420H hydrolyzer/derivatizer.

   Shorter hydrolysis times may be used to optimize recovery of Ser and Thr, which are partially destroyed by HCl hydrolysis, whereas longer times may help with Ile and Val,

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which are slow to cleave. Other modifications of this basic procedure may be necessary to resolve amino acids that are adversely affected by hydrolysis (see Support Protocols for Amino Acid Analysis of Difficult or Unusual Residues).

Derivatize the sample

The following manual protocol is adapted from the Applied Biosystems PTC amino acid analysis instrument manual (version 3.22, May 1989).

8. Dry <20 µg hydrolyzed sample in a clean 6 × 50–mm tube.
9. Add 20 µl of 2:1 (v/v) methanol/DIEA and redry.

   It is possible to substitute ethanol for the methanol and triethylamine (TEA) for the DIEA; reagent by-product peaks in the chromatogram, however, will differ slightly from those shown in Figure 11.9.1.

10. Add 15 µl of 7:2:2 (v/v/v) methanol/DIEA/5% PITC in heptane.
11. Mix samples and incubate 20 min at room temperature.
12. Dry sample in a Speedvac evaporator or 40-ml screw-cap vial. Store at –20°C under argon until ready to analyze.

    Use of instrumentation for automatic PTC derivatization with on-line HPLC analysis (PE Applied Biosystems) reduces variability caused by time-dependent degradation of PTC derivatives.

13. Dissolve the derivatized amino acids in an EDTA-containing transfer buffer compatible with the HPLC system.

    A typical transfer buffer is 29 mM sodium acetate, pH 5.2, containing K_3 EDTA (0.25 mg/ml) which works well for the HPLC separation system shown in Figure 11.9.1. Sample amounts of 0.5 to 1.0 µg protein analysis provide useful results for most PTC-AAA HPLC systems.

    Dissolved samples should be analyzed within 12 to 15 hr. The derivatization reaction is relatively tolerant to variations in pH and molar ratios of reagents to sample, but PTC derivatives may slowly degrade in solution at room temperature.

Analyze the sample

14. Perform quantitative reversed-phase HPLC analysis of the PTC amino acids.

    An example of a typical PTC amino acid separation with UV detection at 254 nm is shown in Figure 11.9.1 along with a description of the chromatography conditions. UV-absorbing by-products from reactions between PITC and other derivatization reagents and sample components (e.g., aniline, phenylthiourea compounds) usually are not problematic provided they are well resolved from amino acid peaks. If increased amounts of by-products are observed, fresh derivatization reagents may be needed. Highly pure solvents and reagents are essential.

    Conditions for separating PTC amino acids by RP-HPLC are well established, and HPLC vendors can provide additional information. Aside from the actual column and HPLC system, important chromatography parameters are temperature, ionic strength and pH of the solvent, and the acetonitrile gradient used for elution. Increasing the temperature and acetonitrile concentration causes all PTC amino acids to elute faster. Asp and Glu are retained longer at low solvent pH and are eluted sooner at higher pH.

Calibrate the system

15. Dilute amino acid Standard H to an appropriate working concentration in water (e.g., 50 to 100 pmol/µl). Store 1 to 2 ml at –20°C.

    The amino acid Standard H from Pierce is convenient for calibration and contains the sixteen amino acids commonly found in protein hydrolysates (Asp, Glu, Ser, Gly, His, Arg,

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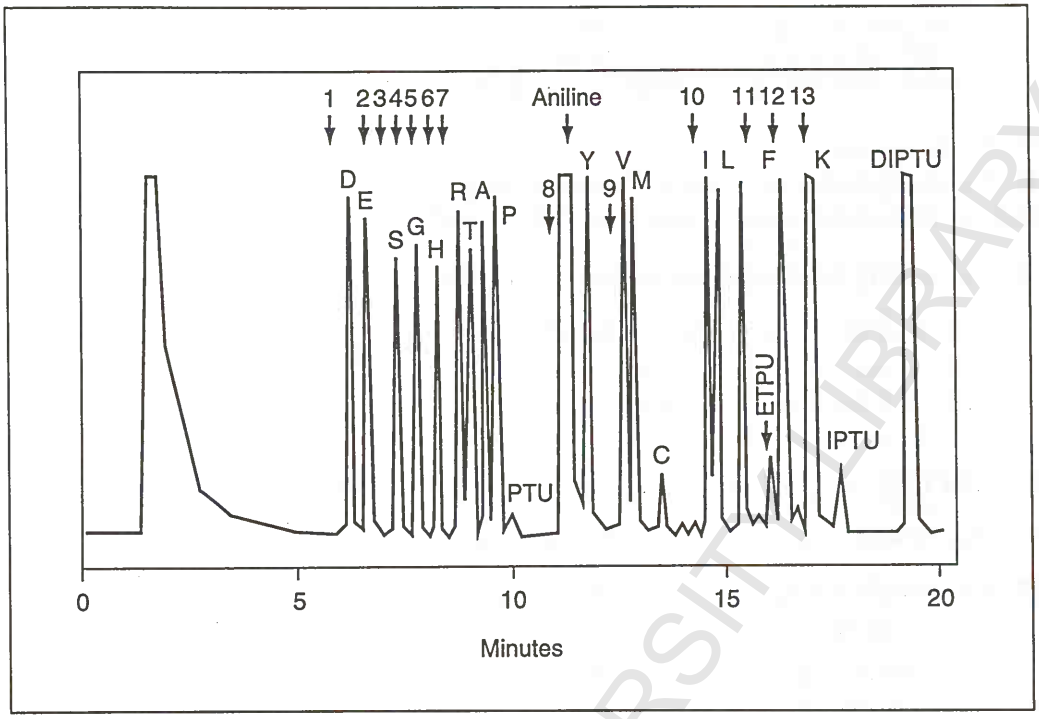

Figure 11.9.1 RP-HPLC of PTC amino acid standards. PTC-derivatized amino acid Standard H (150 pmol; Pierce) was chromatographed using the PE Applied Biosystems Model 130 HPLC and PE Applied Biosystems PTC C_{18} column (2.1 × 220–mm) with a flow rate of 300 µl/min, column temperature of 36°C, and the following gradient: time 0, 5% B; time 10 min, 32% B; time 20 min, 60% B; and time 25 min, 100% B. Solvent A was 50 mM sodium acetate, pH 5.4; solvent B was 31.5 mM sodium acetate containing 70% acetonitrile. The elution positions of sixteen amino acids are identified using the single-letter code (see Table A.1A.1) The elution positions of several other amino acids and PTC derivatization by-products like aniline, phenylthiourea (PTU), ethylphenylthiourea (EPTU), isopropylphenylthiourea (IPTU), and diisopropylphenylthiourea (DIPTU) are also indicated by arrows and numbers: 1, cysteic acid; 2, carboxymethyl cysteine; 3, hydroxyproline; 4, asparagine; 5, glutamine; 6, homoserine; 7, methionine sulfoxide; 8, α-aminobutyric acid; 9, norvaline; 10, pyridylethylcysteine; 11, norleucine; 12, ornithine; 13, tryptophan.

Thr, Ala, Pro, Tyr, Val, Met, Ile, Leu, Phe, and Lys) at the defined concentration of 2.5 µmol/ml plus cystine at 1.25 µmol/ml. Alternatively, quantitative standard solutions may be prepared by weighing individual dry amino acid standards (Pierce) and suspending in 0.1 N HCl to a defined concentration. (See Critical Parameters for further discussion of performance overview using amino acid standards.)

16. Derivatize 1 to 2 nmol (e.g., dry 10 to 20 µl of Standard H at 100 pmol/µl in a 6 × 50–mm tube and follow the PTC derivatization protocol, steps 9 to 13).

17. Perform six to nine PTC amino acid analyses (step 14) with a defined amount of Standard H (e.g., 100 to 300 pmol).

    It is essential that the amount used for calibration fall within the linear response range of the analysis system yet be above background contamination levels. Linear responses are typically obtainable in the range 1 to 5000 pmol; however, background contamination varies from laboratory to laboratory. Background can be problematic at the 50-pmol level and usually will be problematic below 20 pmol.

18. Determine the average peak area (or peak height) for each amino acid from the multiple analyses using an integrator.

    This calculation is greatly facilitated by a computer or inexpensive integrator (such as the Shimadzu C-R6A Chromatopac, which has the ability to store and reprocess a number of analyses).

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19. Calculate a response factor for each amino acid by dividing the average peak area (or peak height) by the picomoles of amino acid analyzed.

$$\text{response factor} = \frac{\text{average peak area}}{\text{pmol amino acid}} \text{ or } \frac{\text{average peak height}}{\text{pmol amino acid}}$$

20. Create a calibration file in the integrator that lists each amino acid by name, retention time, and determined response factor. Set the integrator to divide amino acid peak areas (or peak heights) from unknown samples by the response factors to determine the picomoles of each amino acid in experimental samples.

21. Evaluate the quantitative accuracy of the system every day of use by running at least three standards; update the calibration file as needed. Recalibrate at least once a week.

Determine the linear response range

22. Analyze in replicate five different amounts of Pierce Standard H covering the presumed linear response range (e.g., 1 to 5 nmol).

    To obtain accurate quantification, the detected amount of amino acids must fall within the linear response range of the analyzer. It is important to recognize data that exceed the useful range of measurement (see Anticipated Results).

23. Plot average peak area versus amount analyzed for each amino acid (e.g., see Fig. 11.9.10).

24. Determine the linear response range from the graph and strive to keep all residues in the experimental analyses below the upper limit.

    Background contamination and hydrolysis losses, not sensitivity, are generally the limiting factors at the lower end of the response range.

25. Hydrolyze and analyze in replicate different amounts of a peptide or protein standard of known concentration, calculate the amount of protein analyzed, and plot the results as amount hydrolyzed versus amount recovered.

Determine reproducibility

26. Monitor the reproducibility of residue retention times and standard amino acid peak areas (or peak heights) in the analysis system (see Anticipated Results) by performing several analyses of a defined amount of Pierce Standard H (e.g., 100 to 300 pmol) as described for instrument calibration.

27. Compile retention time and peak area/height data for about six analyses.

28. Calculate the reproducibility (i.e., precision) of retention times and peak areas (or peak heights) as the relative standard deviation (RSD) or % standard deviation about the mean for each amino acid. Calculate average reproducibility by averaging the RSD values for all amino acids measured.

    Reproducibility (i.e., precision) not only is dependent on instrument performance but also is related to amount of sample analyzed, efficiency of PTC derivatization, background contamination levels, pipetting accuracy, and efficiency of hydrolysis for peptides and proteins. In general, variability increases with decreased sample amount and increased background. Reproducible performance must be demonstrated to justify use of the analysis system!

29. Perform several analyses of a defined amount of a standard peptide or protein hydrolysate and calculate the RSD for average peak area (or peak height) for each amino acid.

30. Repeat the analyses and statistical calculations every week over several weeks and months to demonstrate the long-term reproducibility of the system.

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Acceptable PTC-AAA reproducibility is reflected by average RSD values of ~0.3% for retention times and 6 to 7% for peak areas with either Standard H (150-pmol level) or standard proteins (300-ng level). Lower RSD values are attainable; higher RSD values should be avoided.

Verify system performance with a standard peptide or protein

31. Every day when in use, verify the accuracy of the AAA system: hydrolyze, derivatize, and analyze in duplicate a peptide or protein standard.

Any peptide or protein of known composition can be used following quantitation—e.g., insulin, myoglobin, or lysozyme (Sigma). A convenient quantitative standard for this purpose is a 7% solution of bovine serum albumin available from NIST (formerly the National Bureau of Standards).

Calculate amino acid composition in mole %

32. Using data from chromatographic peaks that are accurately identified and integrated and a spreadsheet (e.g., Fig. 11.9.2), enter pmol analyzed for each amino acid (Column B).

33. Sum the total pmol of amino acids in the analysis (B27).

34. Divide pmol of each residue by the sum total (Column B) and multiply by 100. Tabulate results as mole % (Column C).

A mole % calculation example using PTC-AAA data from the analysis of bovine serum albumin is shown in Figure 11.9.3. This information can be used with PROPSEARCH and

	A	B	C
1	DATE RUN	3/31/96	
2	TYPE IN ID#		
3	NAME AND METHOD		
4	TOTAL VOL IN UL		
5	VOL HYD ul		
6	RATIO APPLIED		
7			
8			COMPOSITION
9	AMINO ACID	pmol analyzed	MOLE %
10	ASP (D)		=(B10/B27)
11	GLU (E)		=(B11/B27)
12	SER (S)		=(B12/B27)
13	GLY (G)		=(B13/B27)
14	HIS (H)		=(B14/B27)
15	ARG (R)		=(B15/B27)
16	THR (T)		=(B16/B27)
17	ALA (A)		=(B17/B27)
18	PRO (P)		=(B18/B27)
19	TYR (Y)		=(B19/B27)
20	VAL (V)		=(B20/B27)
21	MET (M)		=(B21/B27)
22	ILE (I)		=(B22/B27)
23	LEU (L)		=(B23/B27)
24	PHE (F)		=(B24/B27)
25	LYS (K)		=(B25/B27)
26			
27	TOTAL PMOL ANALYZED =	=SUM(B10:B25)	=SUM(C10:C25)

Figure 11.9.2 Spreadsheet for calculating mole %. Microsoft Excel data entry commands are shown.

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ExPASy to search databases to provide information about the identity of the peptide or protein (see Support Protocol 19).

Calculate amino acid composition in residues per molecule for unknown sample

35a. For a sample for which the approximate molecular weight is known (e.g., from SDS-PAGE; *UNIT 10.1*), enter the approximate molecular weight (B28) into a spreadsheet, e.g., Figure 11.9.4.

36a. Enter the µl volume hydrolyzed (B5) and the fraction (ratio) of the amount applied to the analyzer that was actually analyzed (B6).

The amount analyzed is a defined fraction of the amount applied and depends upon how the instrument is set up.

37a. Enter pmol analyzed for each amino acid (B9 to B24).

38a. Divide the molecular weight by 112 (assumed average residue weight) to estimate the total residues per molecule (B30).

39a. Sum total pmol analyzed (column B). Divide total pmol analyzed (B26) by total residues per molecule (B30) to estimate pmol protein analyzed (D29).

40a. Divide pmol each amino acid (column B) by pmol protein (D29) to estimate residues per molecule (column C).

	A	B	C
1	DATE RUN	3/31/96	
2	TYPE IN ID#		
3	NAME AND METHOD	BSA	
4	TOTAL VOL IN UL		
5	VOL HYD ul	10	
6	RATIO APPLIED	1.0	
7			
8			COMPOSITION
9	AMINO ACID	pmol analyzed	MOLE %
10	ASP (D)	1606.1	10.67%
11	GLU (E)	2251.26	14.96%
12	SER (S)	760.99	5.06%
13	GLY (G)	545.79	3.63%
14	HIS (H)	469.49	3.12%
15	ARG (R)	637.16	4.23%
16	THR (T)	887.22	5.89%
17	ALA (A)	1316.07	8.74%
18	PRO (P)	832.03	5.53%
19	TYR (Y)	621.56	4.13%
20	VAL (V)	967.31	6.43%
21	MET (M)	112.87	0.75%
22	ILE (I)	298.79	1.98%
23	LEU (L)	1844.82	12.26%
24	PHE (F)	799.26	5.31%
25	LYS (K)	1102.34	7.32%
26			
27	TOTAL PMOL ANALYZED =	15053.06	100.00%

Figure 11.9.3 Calculation example—mole %. PTC-AAA data from the analysis of bovine serum albumin is shown.

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	A	B	C	D	E	F
1	DATE RUN	3/31/96				
2	TYPE IN ID#					
3	NAME AND METHOD	Unknown sample				
4						
5	AMOUNT HYD					
6	RATIO APPLIED					
7			EXPERIMENTAL	COMPOSITION		
8	AMINO ACID	pmol analyzed	RESIDUES	MOLE %	RESIDUE WT.	SUM RESIDUE WTS.
9	ASP (D)		=B9/D29	=(B9/B26)	115.09	=C9*E9
10	GLU (E)		=B10/D29	=(B10/B26)	129.12	=C10*E10
11	SER (S)		=B11/D29	=(B11/B26)	87.08	=C11*E11
12	GLY (G)		=B12/D29	=(B12/B26)	57.05	=C12*E12
13	HIS (H)		=B13/D29	=(B13/B26)	137.14	=C13*E13
14	ARG (R)		=B14/D29	=(B14/B26)	156.19	=C14*E14
15	THR (T)		=B15/D29	=(B15/B26)	101.10	=C15*E15
16	ALA (A)		=B16/D29	=(B16/B26)	71.08	=C16*E16
17	PRO (P)		=B17/D29	=(B17/B26)	97.12	=C17*E17
18	TYR (Y)		=B18/D29	=(B18/B26)	163.18	=C18*E18
19	VAL (V)		=B19/D29	=(B19/B26)	99.07	=C19*E19
20	MET (M)		=B20/D29	=(B20/B26)	131.19	=C20*E20
21	ILE (I)		=B21/D29	=(B21/B26)	113.16	=C21*E21
22	LEU (L)		=B22/D29	=(B22/B26)	113.16	=C22*E22
23	PHE (F)		=B23/D29	=(B23/B26)	147.18	=C23*E23
24	LYS (K)		=B24/D29	=(B24/B26)	128.17	=C24*E24
25						
26	TOTAL PMOL ANALYZED =	=SUM(B9:B24)	CALCULATED	=SUM(F9:F24)+18	AV. RES. WT.	=D26/B30
27			MW PROTEIN			
28	estimated molecular weight=					
29			PMOL PROTEIN	=B26/B30	PMOL/ul	=D29/B6/B5
30	ESTIMATED TOTAL RESIDUES	=B28/112	ANALYZED			
31	(ASSUMING AVE RESIDUE WT=112)					
32			UG PROTEIN	=(D29*D26)/1000000	ug/ul	=D32/B6/B5
33	EXP TOTAL RESIDUES	=SUM(C9:C24)	ANALYZED			

Figure 11.9.4 Spreadsheet for calculating residues per molecule for unknown samples. Microsoft Excel data entry commands are shown.

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41a. To calculate the original sample concentration (F29) in pmol/µl, divide the pmol protein analyzed (D29) by the µl volume hydrolyzed (B5) and the ratio of the applied amount that was actually analyzed (B6).

To simplify the ratio applied, the integrator can be set to normalize the pmol values to 100% analyzed.

42a. Calculate mole % in column D by dividing by total pmol analyzed (B26).

43a. Enter the average residue weight for each amino acid in column E. Calculate the sum of the residue weights in column F by multiplying residue values (column C) by residue weights (column E).

44a. Calculate the molecular weight of the sample (D26) by summing F9 through F24 and adding 18 for water.

45a. Calculate µg protein analyzed (D32) by multiplying pmol analyzed (D29) by the molecular weight (D26) and by 10^{-6}.

46a. Calculate the original sample concentration (F32) in µg/µl: divide the µg amount analyzed (D32) by the µl volume hydrolyzed (B5) and the ratio of the applied amount that was actually analyzed (B6).

A calculation of the residues per molecule for PTC-AAA of bovine serum albumin is shown in Figure 11.9.5.

Calculate amino acid composition residues per molecule for known sample

35b. For samples of known mass and composition, use the spreadsheet in Figure 11.9.6 to calculate residues per molecule. Enter the known molecular weight (B6), the µl volume hydrolyzed (B4), and the fraction (ratio) of the amount applied to the analyzer that was actually analyzed (B5).

This method allows sample quantitation from partial compositional data and provides a rapid evaluation of the accuracy of the analysis. The calculation methods outlined in Figures 11.9.2 to 11.9.5 can also be applied to samples of known mass and composition.

The amount analyzed is a defined fraction of the amount applied and depends upon how the instrument is set up.

36b. Enter pmol analyzed for each amino acid (column C) and the known residues per molecule (column B).

37b. Divide pmol of each residue (column C) by the known residue value (column B) to determine pmol protein based on each amino acid (column D).

38b. Estimate the amount analyzed (B27) by averaging all the values in column D.

39b. Discard pmol protein values with unacceptable deviation from the mean (e.g., greater than ±15%).

This is an arbitrary window that can be adjusted (for example, to ±30%) to optimize the number of relevant residues in the recalculated mean (F26).

40b. Calculate the average pmol protein analyzed by averaging the remaining relevant values (F26).

41b. Calculate the experimental composition in residues per molecule (column G) by dividing the amount of each residue by the calculated pmol protein analyzed (F26).

42b. Round off experimental residue values (column G) to integer values (column H).

This is the determined amino acid composition.

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	A	B	C	D	E	F
1	DATE RUN	3/31/96				
2	TYPE IN ID #					
3	NAME AND METHOD	Unknown sample				
4						
5	AMOUNT HYD	10				
6	RATIO APPLIED	1				
7			EXPERIMENTAL	COMPOSITION		
8	AMINO ACID	pmol analyzed	RESIDUES	MOLE %	RESIDUE WT.	SUM RESIDUE WTS.
9	ASP (D)	1606.1	62.9	10.67%	115.09	7236
10	GLU (E)	2251.26	88.1	14.96%	129.12	11379
11	SER (S)	760.99	29.8	5.06%	87.08	2594
12	GLY (G)	545.79	21.4	3.63%	57.05	1219
13	HIS (H)	469.49	18.4	3.12%	137.14	2521
14	ARG (R)	637.16	24.9	4.23%	156.19	3896
15	THR (T)	887.22	34.7	5.89%	101.11	3512
16	ALA (A)	1316.07	51.5	8.74%	71.08	3662
17	PRO (P)	832.03	32.6	5.53%	97.12	3163
18	TYR (Y)	621.56	24.3	4.13%	163.18	3971
19	VAL (V)	967.31	37.9	6.43%	99.13	3754
20	MET (M)	112.87	4.4	0.75%	131.19	580
21	ILE (I)	298.79	11.7	1.98%	113.16	1324
22	LEU (L)	1844.82	72.2	12.26%	113.16	8172
23	PHE (F)	799.26	31.3	5.31%	147.18	4605
24	LYS (K)	1102.34	43.2	7.32%	128.17	5532
25						
26	TOTAL PMOL ANALYZED =	15053.06	CALCULATED	67138	AV. RES. WT.	113.92
27			MW PROTEIN			
28	estimated molecular weight =	66000				
29			PMOL PROTEIN	25.54	PMOL/ul	2.554
30	ESTIMATED TOTAL RESIDUES	589	ANALYZED			
31	(ASSUMING AVE RESIDUE WT=112)					
32			UG PROTEIN	1.71	ug/ul	0.171
33	EXP TOTAL RESIDUES	589.29	ANALYZED			

Figure 11.9.5 Calculation example—residues per molecule for unknown samples. PTC-AAA data from the analysis of bovine serum albumin is shown.

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	A	B	C	D	E	F	G	H	I
1	DATE RUN	3/31/96							
2	TYPE IN								
3	NAME/ID #	KNOWN							
4	VOL HYDROLYZED								
5	RATIO APPLIED								
6	Molecular weight								
7	AMINO ACID	known Composition	pmol analyzed	PMOL PROTEIN	OVER 15%	UNDER 15%	EXP COMP	INT COMP	% INT ERR
8	ASP (D)			=IF(B8=0,FALSE,C8/B8)	=IF(D8<B30,D8,FALSE)	=IF(E8>B29,E8,FALSE)	=C8/(F26)	=ROUND(G8,0)	=IF(B8=0,FALSE,(ABS(B8-H8)/B8)*100)
9	GLU (E)			=IF(B9=0,FALSE,C9/B9)	=IF(D9<B30,D9,FALSE)	=IF(E9>B29,E9,FALSE)	=C9/(F26)	=ROUND(G9,0)	=IF(B9=0,FALSE,(ABS(B9-H9)/B9)*100)
10	SER (S)			=IF(B10=0,FALSE,C10/B10)	=IF(D10<B30,D10,FALSE)	=IF(E10>B29,E10,FALSE)	=C10/(F26)	=ROUND(G10,0)	=IF(B10=0,FALSE,(ABS(B10-H10)/B10)*100)
11	GLY (G)			=IF(B11=0,FALSE,C11/B11)	=IF(D11<B30,D11,FALSE)	=IF(E11>B29,E11,FALSE)	=C11/(F26)	=ROUND(G11,0)	=IF(B11=0,FALSE,(ABS(B11-H11)/B11)*100)
12	HIS (H)			=IF(B12=0,FALSE,C12/B12)	=IF(D12<B30,D12,FALSE)	=IF(E12>B29,E12,FALSE)	=C12/(F26)	=ROUND(G12,0)	=IF(B12=0,FALSE,(ABS(B12-H12)/B12)*100)
13	ARG (R)			=IF(B13=0,FALSE,C13/B13)	=IF(D13<B30,D13,FALSE)	=IF(E13>B29,E13,FALSE)	=C13/(F26)	=ROUND(G13,0)	=IF(B13=0,FALSE,(ABS(B13-H13)/B13)*100)
14	THR (T)			=IF(B14=0,FALSE,C14/B14)	=IF(D14<B30,D14,FALSE)	=IF(E14>B29,E14,FALSE)	=C14/(F26)	=ROUND(G14,0)	=IF(B14=0,FALSE,(ABS(B14-H14)/B14)*100)
15	ALA (A)			=IF(B15=0,FALSE,C15/B15)	=IF(D15<B30,D15,FALSE)	=IF(E15>B29,E15,FALSE)	=C15/(F26)	=ROUND(G15,0)	=IF(B15=0,FALSE,(ABS(B15-H15)/B15)*100)
16	PRO (P)			=IF(B16=0,FALSE,C16/B16)	=IF(D16<B30,D16,FALSE)	=IF(E16>B29,E16,FALSE)	=C16/(F26)	=ROUND(G16,0)	=IF(B16=0,FALSE,(ABS(B16-H16)/B16)*100)
17	TYR (Y)			=IF(B17=0,FALSE,C17/B17)	=IF(D17<B30,D17,FALSE)	=IF(E17>B29,E17,FALSE)	=C17/(F26)	=ROUND(G17,0)	=IF(B17=0,FALSE,(ABS(B17-H17)/B17)*100)
18	VAL (V)			=IF(B18=0,FALSE,C18/B18)	=IF(D18<B30,D18,FALSE)	=IF(E18>B29,E18,FALSE)	=C18/(F26)	=ROUND(G18,0)	=IF(B18=0,FALSE,(ABS(B18-H18)/B18)*100)
19	MET (M)			=IF(B19=0,FALSE,C19/B19)	=IF(D19<B30,D19,FALSE)	=IF(E19>B29,E19,FALSE)	=C19/(F26)	=ROUND(G19,0)	=IF(B19=0,FALSE,(ABS(B19-H19)/B19)*100)
20	ILE (I)			=IF(B20=0,FALSE,C20/B20)	=IF(D20<B30,D20,FALSE)	=IF(E20>B29,E20,FALSE)	=C20/(F26)	=ROUND(G20,0)	=IF(B20=0,FALSE,(ABS(B20-H20)/B20)*100)
21	LEU (L)			=IF(B21=0,FALSE,C21/B21)	=IF(D21<B30,D21,FALSE)	=IF(E21>B29,E21,FALSE)	=C21/(F26)	=ROUND(G21,0)	=IF(B21=0,FALSE,(ABS(B21-H21)/B21)*100)
22	PHE (F)			=IF(B22=0,FALSE,C22/B22)	=IF(D22<B30,D22,FALSE)	=IF(E22>B29,E22,FALSE)	=C22/(F26)	=ROUND(G22,0)	=IF(B22=0,FALSE,(ABS(B22-H22)/B22)*100)
23	LYS (K)			=IF(B23=0,FALSE,C23/B23)	=IF(D23<B30,D23,FALSE)	=IF(E23>B29,E23,FALSE)	=C23/(F26)	=ROUND(G23,0)	=IF(B23=0,FALSE,(ABS(B23-H23)/B23)*100)
24									
25	TOTAL #AA	=SUM(B8:B23)			NEW ESTIMATE	PMOL PROTEIN			
26	ESTIMATED AMOUNT	ANALYZED			WITHIN 15%	=AVERAGE(F8:F23)		AVERAGE %	
27	PMOL PROTEIN	=AVERAGE(D8:D23)						ERROR	=AVERAGE(I8:I23)
28	15% OF PROTEIN	=B27*0.15		TOTAL PMOL	AMINO ACID	=SUM(C8:C23)			
29	-15% OF PROTEIN	=B27-B28		TOTAL	PMOL HYDROL	=F26/B5	Total ugrams	=(F29*B6)/1000000	
30	+15% OF PROTEIN	=B27+B28			CONC pmol/µl	=F29/B4	CONC µg/µl	=H29/B4	

Figure 11.9.6 Spreadsheet for calculating residues per molecule for known samples. Microsoft Excel data entry commands are shown.

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	A	B	C	D	E	F	G	H	I
1	DATE RUN	3/31/96							
2	TYPE IN								
3	NAME/ID #	KNOWN (NBS-BSA)							
4	VOL HYDROLYZED	10							
5	RATIO APPLIED	1.00							
6	Molecular weight	66328							
7	AMINO ACID	known Composition	pmol analyzed	PMOL PROTEIN	OVER 15%	UNDER 15%	EXP COMP	INT COMP	% INT ERR
8	ASP (D)	54	1606.1	29.74	29.74	29.74	56.9	57	5.56
9	GLU (E)	79	2251.26	28.50	28.50	28.50	79.7	80	1.27
10	SER (S)	28	760.99	27.18	27.18	27.18	26.9	27	3.57
11	GLY (G)	16	545.79	34.11	FALSE	FALSE	19.3	19	18.75
12	HIS (H)	17	469.49	27.62	27.62	27.62	16.6	17	0.00
13	ARG (R)	24	637.16	26.55	26.55	26.55	22.6	23	4.17
14	THR (T)	34	887.22	26.09	26.09	26.09	31.4	31	8.82
15	ALA (A)	46	1316.07	28.61	28.61	28.61	46.6	47	2.17
16	PRO (P)	28	832.03	29.72	29.72	29.72	29.5	30	7.14
17	TYR (Y)	19	621.56	32.71	FALSE	FALSE	22.0	22	15.79
18	VAL (V)	36	967.31	26.87	26.87	26.87	34.2	34	5.56
19	MET (M)	4	112.87	28.22	28.22	28.22	4.0	4	0.00
20	ILE (I)	14	298.79	21.34	21.34	FALSE	10.6	11	21.43
21	LEU (L)	61	1844.82	30.24	30.24	30.24	65.3	65	6.56
22	PHE (F)	27	799.26	29.60	29.60	29.60	28.3	28	3.70
23	LYS (K)	59	1102.34	18.68	18.68	FALSE	39.0	39	33.90
24									
25	TOTAL #AA	546			NEW ESTIMATE	PMOL PROTEIN			
26	ESTIMATED AMOUNT	ANALYZED			WITHIN 15%	28.25		AVERAGE %	
27	PMOL PROTEIN	27.86						ERROR	8.65
28	15% OF PROTEIN	4.18		TOTAL PMOL	AMINO ACID	15053.06			
29	-15% OF PROTEIN	23.68		TOTAL	PMOL HYDROL	28.25	Total ugrams	1.87	
30	+15% OF PROTEIN	32.04			CONC pmol/µl	2.825	CONC µg/µl	0.187	

Figure 11.9.7 Calculation example—residues per molecule for known samples. PTC-AAA data from the analysis of bovine serum albumin is shown.

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43b. Calculate % error for each residue (column I) based on integer values (column H).

44b. Calculate the original sample concentration in pmol/µl (F30) by dividing the pmol protein analyzed (F26) by the ratio applied (B5) and the µl volume hydrolyzed (B4).

45b. Calculate total µg hydrolyzed (H29) by multiplying [pmol protein analyzed (F26)] × [ratio applied (B5)] × [molecular weight (D26)] × [10^{-6}].

46b. Calculate the original sample concentration in µg/µl (H30): divide total µg protein hydrolyzed (H29) by the µl volume hydrolyzed (B4).

A calculation of residues per molecule for PTC-AAA of bovine serum albumin is shown in Figure 11.9.7.

Determine compositional accuracy

47. Evaluate compositional accuracy by calculating average compositional % error relative to the known composition of the sample (i.e., known from the amino acid sequence).

This value usually excludes Trp and Cys but reflects all other residue values unadjusted for partial destruction, incomplete hydrolysis, or contamination.

48. Calculate % error per amino acid after rounding the experimentally observed number of residues to the nearest integer (see Figs. 11.9.6 and 11.9.7).

$$\%\ \text{error} = 100 \times \frac{\text{known number of residues} - \text{experimental number of residues}}{\text{known number of residues}}$$

$$\text{average \% compositional error} = \frac{\Sigma\ \%\ \text{error for } n \text{ amino acids}}{n}$$

Usually, n = 16.

49. Check compositional and quantitative accuracy daily.

Strive to maintain compositional and quantitative accuracy within 10% of the known values. AAA requires system performance verification with a standard peptide or protein of defined concentration and composition.

SUPPORT PROTOCOLS FOR SAMPLE PREPARATION

The purpose of sample preparation is to produce homogeneous, salt- and detergent-free samples. Samples for amino acid analysis (AAA) may be desalted by one of several methods such as microdialysis (see Support Protocol 3), organic solvent precipitation (see Support Protocol 4 or 5), RP-HPLC (see Support Protocol 1), and centrifugation using microseparation concentrators (Amicon; see Support Protocol 2). Recovery from these procedures can be variable and sample dependent and can complicate the process of determining the concentration of stock preparations. Remember to check for and record sample volume changes that may occur during desalting and concentration steps.

Sample Preparation by RP-HPLC

SUPPORT PROTOCOL 1

Additional Materials (also see Basic Protocol)

Solvent, e.g., trifluoroacetic acid (Sequenal-grade TFA, Pierce)/acetonitrile (UV HPLC-grade, Burdick and Jackson)

RP-HPLC column, minicolumn, or disposable cartridge support (e.g., C_4 or C_{18} support with 300-Å pore size, Vydac)

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Desalt samples by RP-HPLC (see *UNIT 11.6*) using a column and solvent appropriate for peptides and proteins. Classic aqueous trifluoroacetic acid (TFA)/acetonitrile solvents, are excellent for RP-HPLC desalting purposes. A variety of standard HPLC columns, minicolumns, and disposable cartridge style supports may be useful.

SUPPORT PROTOCOL 2

Sample Preparation by Microseparation Concentrators

CentriPrep, Centricon, and Microcon concentrators (Amicon) are particularly useful for simultaneously concentrating samples and exchanging solvents, and they are available in different volumes and exclusion limits (specifications and detailed instructions for use are supplied by the vendor). Apply the sample to the tube, centrifuge until the volume is appropriately reduced, add volatile solvent (see Basic Protocol, annotation to step 6 for examples), and repeat centrifugation. Repeat the exchange two to three times, depending on the original salt concentration.

SUPPORT PROTOCOL 3

Sample Preparation by Microdialysis

Microdialysis can be used to prepare 100- to 200-µl samples in a method adapted from Orr et al. (1995).

Additional Materials (also see Basic Protocol)

Solvent (e.g., see Basic Protocol, step 6 annotation)
1.5-ml clear microcentrifuge tube
Spectra/Por dialysis membrane (Spectrum Medical), rehydrated

1. Cut the cap off a microcentrifuge tube (~1.5 ml) with scissors and then cut the tube in two.
2. Place the cap upside down on the bench and pipet the peptide or protein into the "cup" of the cap, which typically holds ~200 µl.

   If the sample contains a high concentration of salt (e.g., 2 to 8 M urea or 6 M guanidine), apply ≤100 µl in order to leave room for volume expansion during dialysis.

3. Cut open a small piece of rehydrated Spectra/Por dialysis membrane of appropriate exclusion limit. Cover the cap and sample with a single layer of membrane and secure with the top piece of the cut tube.
4. Float the cap in a beaker of solvent (200 to 500 ml) and cover the beaker; dialyze 30 min with vigorous mixing. Change solvent and repeat three times.
5. Remove cap and collect the sample by puncturing the membrane with a micropipet.

SUPPORT PROTOCOL 4

Sample Preparation by Acetone Precipitation for Proteins

This procedure is suitable for recovering proteins from most aqueous solvents and from SDS-containing buffers. It is not recommended for proteins dissolved in urea or guanidine or for peptides. Practice the precipitation procedure with a readily available protein before attempting use with a precious sample. This procedure is useful for removing SDS from protein preparations after electrophoretic purification and electroelution from polyacrylamide gels; it can also be used for concentrating samples for Edman sequence analysis and/or proteolytic digestion.

Additional Materials (also see Basic Protocol)

Acetone (Burdick and Jackson)
Ethyl acetate (Burdick and Jackson)
N-ethylmorpholine (Sequenal grade, Pierce)

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HCl (ULTREX II ultrapure reagent, J.T. Baker)
Trifluoroacetic acid (Sequenal-grade TFA, Pierce)
Centrifuge

1. Ensure that the sample volume is in the range of 20 µl to 2 ml.

   For <10 µg protein, the volume should be reduced to <500 µl. This can be accomplished by partial drying in a Speedvac evaporator or by several extractions with excess ethyl acetate.

2. Add 2 vol acetone and vortex.

   The precipitated protein will be visible, usually as a flocculent, white particulate material. A magnifying glass can help in visualizing the precipitate.

3. If no precipitate is apparent, add ~0.5 vol ethyl acetate; vortex again.

4. If still no precipitate is apparent, change pH slightly by adding a few microliters of *N*-ethylmorpholine to raise the pH or HCl to lower the pH. Check pH before and after additions by pipetting ~1 µl to an appropriate range of pH paper.

   The protein will precipitate at its isoelectric pH.

5. Centrifuge and carefully remove the supernatant. Save the supernatant if the location of the sample is questionable.

6. For amino acid or sequence analysis, desalt the sample (e.g., wash the pellet carefully with a small volume of 67% acetone, blow dry with a gentle stream of nitrogen, and suspend in 100% TFA for necessary transfers).

   For proteolytic digestion, the pellet usually need not to be washed.

Peptide Sample Preparation by Ether Precipitation

SUPPORT PROTOCOL 5

This procedure is suitable for precipitating peptides from organic acids and is used routinely for concentrating synthetic peptides from the trifluoroacetic acid cleavage cocktail following 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis. It can be used to desalt peptides in nonvolatile buffers but is not recommended for removing detergents like SDS. Take appropriate safety precautions when using this technique; ether is highly flammable, and a refrigerated centrifuge and fume hood are recommended. Practice the precipitation procedure with 5- to 25-µg amounts of a standard peptide before attempting use with a precious sample. Maintain cold solution temperatures for best results.

Additional Materials (also see Basic Protocol)

75% trifluoroacetic acid (Sequenal grade TFA, Pierce)
Ether, ice-cold

6 × 50–mm tube
Centrifuge, 4°C

CAUTION: Ether is highly flammable; work with a fume hood.

1. Vacuum dry an aliquot of the peptide preparation in a 6 × 50–mm tube. Redissolve the peptide in 50 to 200 µl of 75% TFA and cool to 4°C on ice.

   Alternatively, the peptide preparation can be diluted with 100% TFA to a final concentration of 75% TFA and cooled to 4°C. Keep final sample volumes small (e.g., ~200 µl).

2. Add 3 vol ice-cold ether, mix, and incubate 15 to 30 min on ice or in a –20°C freezer.

   Flocculent peptide is usually visible above 20 µg but increasingly difficult to see below 20 µg.

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3. Centrifuge 5 min at 4°C.
4. Decant supernant immediately while cold; vacuum dry the pellet.

   For amounts below ~5 µg, a pellet may not be visible. Hydrolyze and analyze anyway (see Basic Protocol, steps 7 to 14).
5. Repeat steps 1 to 4 as needed for very salty samples.

   Typically very little precipitate is visible in the tube following vacuum drying. Substantial precipitate suggests excess salt remains.

SUPPORT PROTOCOLS FOR HYDROLYSIS

The hydrolysis process has a major impact on the quality of results from amino acid analysis (AAA). Liquid-phase (see Support Protocol 8) and vapor-phase (see Support Protocol 7) HCl hydrolysis procedures are both popular as standard, complete hydrolysis methods. Examples of each method are presented. However, vapor-phase methods receive more wide-spread use. Samples can also be hydrolyzed on PVDF membranes (see Support Protocol 9) or using a microwave hydrolysis system (see Support Protocol 10). Vapor-phase and liquid-phase hydrolysis require specially prepared tubes and vials (see Support Protocol 6). For more information regarding the influence of various hydrolysis conditions, see the AAA studies sponsored by the Association of Biomolecular Resource Facilities, particularly Tarr et al. (1991), Strydom et al. (1992), and Yücksel et al. (1995).

SUPPORT PROTOCOL 6

Preparation of Hydrolysis Tubes and Vials

1. Use 6 × 50–mm hydrolysis tubes for either vapor- or liquid-phase hydrolyses (see Support Protocols 7 and 8).
2. Rinse the tubes at least five times with water followed by five times with methanol (Burdick and Jackson) and then pyrolyze by heating 5 hr at 500°C. Clean tubes batchwise.
3. Store clean tubes in a dust-free, closed container. Discard used tubes—do not reuse.
4. Use clean bulge-modified 40-ml screw-cap vials (see Basic Protocol, step 4) for either vapor- or liquid-phase hydrolyses (see Support Protocols 7 and 8). Clean the modified vials with soap, rinse exhaustively with water and then methanol, and dry. Reuse bulge vials but always clean first.

SUPPORT PROTOCOL 7

Vapor-Phase HCl Hydrolysis of Samples

Additional Materials (also see Basic Protocol)

6 N HCl (Sequenal grade, Pierce)
Phenol (Aldrich)
Diamond pencil

1. Vacuum dry purified, salt-free peptide/protein samples in clean 6 × 50–mm tubes (~0.5 to 5 µg/tube) using a Speedvac evaporator. Use a diamond pencil to etch identifying marks on each tube.
2. Place 6 × 50–mm sample tube in a bulge modified 40-ml screw-cap vial containing ~300 µl of 6 N HCl plus a crystal of phenol (~1 to 2 mg). Seal the vial with a modified Mininert slide valve (see Basic Protocol, step 3).

   Phenol serves as a scavenger to prevent halogenation of Tyr. The bulge modification of the screw-cap vial prevents HCl condensate from running down the inside wall of the vial into the 6 × 50–mm sample tube during hydrolysis.

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3. Evacuate briefly (15 to 20 sec) then flush with argon or nitrogen 1 to 2 sec. Repeat this alternating process three times, closing the slide valve (red position in) on the fourth vacuum step.
4. Heat 1 hr at 150°C. Following hydrolysis, release the pressure within a fume hood by pointing the cap away from your face and pushing the slide valve open. Perform time course hydrolyses for 1, 2, and 4 hr if desired.
5. Transfer the sample tubes to a clean, unmodified 40-ml vial and vacuum dry. Store at –20°C under argon until ready to perform AAA.

Liquid-Phase HCl Hydrolysis of Samples

SUPPORT PROTOCOL 8

Additional Materials (also see Basic Protocol)

6 N HCl (Sequenal grade, Pierce) containing 1% (v/v) phenol (Aldrich)
Diamond pencil

1. Follow steps 1 and 2 for manual vapor-phase hydrolysis (see Support Protocol 7) but add 20 µl of 6 N HCl containing 1% phenol directly to each sample. Seal the vial with a modified Mininert slide valve (see Basic Protocol, step 3).
2. Evacuate 15 to 20 sec and flush 1 to 2 sec with argon or nitrogen. Repeat three times. Watch for bubbles during the vacuum phase; avoid bumping when switching to argon flush. Close the slide valve (red position in) on the last vacuum step.
3. Heat 20 to 24 hr at ~110°C. Following hydrolysis, release the pressure within a fume hood by pointing the cap away from your face and pushing the slide valve open. Perform time course hydrolyses for 24, 48, and 72 hr if desired.
4. Transfer the sample tubes to a clean, unmodified 40-ml vial and vacuum dry. Store at –20°C under argon until ready to perform AAA.

Hydrolysis of Samples on PVDF Membranes

SUPPORT PROTOCOL 9

Additional Materials (also see Basic Protocol)

5% acetic acid/50% methanol (Burdick and Jackson)
50% acetonitrile (UV HPLC grade; Burdick and Jackson) *or* 50% methanol (Burdick and Jackson)
Polyvinylidene difluoride (PVDF) membrane (PE Applied Biosystems, Millipore)
ProSpin cartridge membrane (PE Applied Biosystems; optional)
PVC gloves, powder-free
Additional reagents and equipment for vapor-phase hydrolysis (see Support Protocol 7) or liquid-phase hydrolysis (see Support Protocol 8)

1. Apply samples to PVDF membranes by electroblotting, by centrifuging onto a ProSpin cartridge membrane, by absorbing, or by spotting with a micropipettor.

   Be aware that variable amino acid analysis results are frequently obtained from samples spotted or absorbed on PVDF membrane, presumably due to difficult-to-extract background contamination within the membrane (Tarr et al., 1991; Mahrenholz et al., 1996).

2. Wear powder-free PVC gloves, maintain clean technique, and use clean glassware and reagents. Cut PVDF membrane to fit into a 6 × 50–mm hydrolysis tube. Prior to hydrolysis wash briefly in 5% acetic acid/50% methanol to remove any excess stain. Prepare blank control pieces of PVDF from membrane treated as for the experimental samples.

   Forceps are useful for handling the small pieces of membrane.

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3. Hydrolyze control and experimental PVDF samples using standard vapor- or liquid-phase HCl hydrolysis methods (see Support Protocol 7 or 8).

4. Following hydrolysis, add 100 µl of 50% acetonitrile or 50% methanol to each tube, incubate 10 min, then transfer the solution to a second 6 × 50–mm tube. Repeat the extraction once and vacuum dry the combined extracts.

   If necessary, samples extracted from PVDF membranes can be stored at –20°C under argon until ready to perform AAA.

5. PTC derivatize and analyze as usual (see Basic Protocol).

   Results from control samples can be subtracted from experimental samples. However, because background contamination is extremely variable, subtracting background does not always improve the results. Higher quality results are more readily obtained with sample amounts >1 µg (Gharahdaghi et al., 1992).

SUPPORT PROTOCOL 10

Microwave Hydrolysis of Samples

Microwave hydrolysis can reduce the time required to perform HCl vapor-phase hydrolysis. The following procedure was adapted from Hsi and Yamane (1994) and is based on the use of a CEM MDS 2000 Microwave Sample Preparation System equipped with a Teflon vapor-phase hydrolysis vessel and special microvials. The instrumentation is expensive compared with kitchen microwaves but is specially designed for vapor-phase hydrolysis for amino acid analysis. For a description of this microwave instrument, see Engelhart (1990).

Additional Materials (also see Basic Protocol)

6 N HCl
Microwave Sample Preparation System (CEM) consisting of:
  Microwave oven
  Teflon vapor-phase hydrolysis vessel
  Microvials

1. Dry samples in microvials; place up to ten vials in the Teflon vapor-phase hydrolysis vessel.

2. Add 5 ml of 6 N HCl to the hydrolysis vessel, flush with nitrogen, and seal.

3. Microwave hydrolyze 20 min at 150°C.

4. Vacuum dry, PTC derivatize, and analyze (see Basic Protocol, steps 8 to 14).

SUPPORT PROTOCOLS FOR AMINO ACID ANALYSIS OF DIFFICULT OR UNUSUAL RESIDUES

The Basic Protocol provides for analysis of only sixteen of the common amino acids. Difficult and unusual residues—e.g., cysteine, tryptophan, and phosphoamino acids—are often adversely affected by standard hydrolysis, so special hydrolysis conditions are required. In addition, chromatographic conditions may need to be modified to optimize resolution, as in the case of hydoroxyproline which may coelute with other residues. The response factors for these residues must be carefully determined using the appropriate standards (see Support Protocol 11).

Quantification of cysteine is difficult because hydrolysis in 6 N HCl destroys the residue and partially destroys the disulfide-linked dimeric form of the residue, cystine. This sulfur-containing residue must be modified for accurate measurement; two useful modification methods that receive widespread use are oxidative hydrolysis (see Support

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Protocol 12) and pyridylethylation (see Support Protocol 13). A comparison of the performance of these and other methods for cysteine analysis can be found in Strydom et al. (1993).

Tryprophan is destroyed by standard HCl hydrolysis and is even more challenging to quantify than cysteine. Two successful alternate hydrolysis strategies for quantifying tryptophan are vapor-phase HCl hydrolysis in the presence of dodecanethiol (see Support Protocol 13) and hydrolysis with 4 N methanesulfonic acid (see Support Protocol 15). A comparison of the performance of these and other methods for tryptophan analysis can be found in Strydom et al. (1993).

Phosphoamino acid analysis is not yet routine for most laboratories that perform amino acid analysis (AAA), and no current AAA methodology allows quantification of phosphoamino acids in a single analysis in conjunction with common amino acids (Yücksel et al., 1994). Hydrolysis is the most critical part of the analytical process because of the acid lability of the phosphate moiety. Optimal hydrolysis conditions (see Support Protocols 16 and 17) must be determined for each sample peptide or protein. Hydrolysis conditions do not generally provided for quantitative cleavage of peptide bonds and often produce short peptides that may interfere with the measurement of other amino acids. The peptide or protein concentration must be verified in a separate analysis using standard conditions for complete hydrolysis.

Hydroxyproline can be hydrolyzed and derivatized using standard conditions, but it may coelute with other residues such as glutamic acid, so chromatography conditions may need to be modified to optimize resolution of hydroxyproline in HPLC (see Support Protocol 18).

SUPPORT PROTOCOL 11

Determining Response Factors for Difficult Residues

Determine response factors for difficult residues such as cysteic acid (Cya) and tryptophan (Trp) from protein hydrolysates.

1. Perform the appropriate hydrolysis (see Basic Protocol, step 7) with more than one standard peptide or protein containing the residue of interest, e.g., oxidative hydrolysis for Cya (see Support Protocols 12 and 13) and hydrolysis with methanesulfonic acid (see Support Protocol 15) or dodecanethiol (see Support Protocol 14) for Trp .
2. Determine the amount of standard protein analyzed (see Figs. 11.9.6 and 11.9.7).
3. Divide the peak area (or peak height) of the amino acid of interest (e.g., Cya or Trp) by the amount of protein analyzed.
4. Finally, divide by the known number of residues of interest in the standard protein (e.g., for lysozyme, determine the response factor for Cya by dividing by 8).
5. Use the hydrolysate-derived response factor for converting Trp and Cya peak area (or peak height) to picomoles of amino acid analyzed.
6. Interpret data for the other residues using response factors derived from the routine quantitative amino acid standard (e.g., Standard H).

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SUPPORT PROTOCOL 12

Oxidative Hydrolysis for Quantification of Cysteine

Oxidation of cysteine/cystine to cysteic acid (Cya) by hydrolysis in the presence of dimethylsulfoxide (DMSO) is a simple and convenient method of Cys quantification. The method was originally introduced by Spencer and Wold (1969) and more recently demonstrated to be effective in PTC-AAA (West and Crabb, 1992).

Additional Materials (also see Basic Protocol)

2% (v/v) dimethylsulfoxide (DMSO; HPLC grade, Aldrich)
2% (w/v) diisopropylethylamine (DIEA; PE Applied Biosystems)
Cysteic acid (Sigma)
Standard protein containing cysteine, e.g., cytochrome *c*, or β-lactoglobulin, lysozyme, PTC-derivatized

Additional reagents and equipment for vapor-phase hydrolysis (see Support Protocol 7) and determining response factor for cysteic acid (see Support Protocol 11)

1. Subject the peptide or protein to manual vapor-phase HCl hydrolysis (see Support Protocol 7) *except* add 2% (v/v) DMSO to the 6 N HCl and omit the phenol.
2. Follow the manual PTC derivatization protocol (see Basic Protocol, steps 8 to 11) and vacuum dry.
3. Dissolve the derivatized sample in EDTA-containing transfer buffer containing 2% (w/v) DIEA and incubate 10 min at room temperature prior to HPLC analysis.

   This step was developed as a vapor-phase "bubbling" step during automatic PTC derivatization and helps neutralize the negative charge on Cya through ion pairing, resulting in increased Cya retention time (Hsi and Yamane, 1994). Optimization of HPLC analysis of PTC-Cya (see Fig. 11.9.1) may also require lowering the solvent pH a few tenths of a unit to increase its retention.

4. Determine response factors for cysteic acid (see Support Protocol 11) by oxidative hydrolysis and PTC-AAA of standard cysteine-containing proteins such as cytochrome *c* (2 Cys/mol), β-lactoglobulin (5 Cys/mol), and lysozyme (8 Cys/mol).

   A cysteic acid standard is useful for optimizing chromatography, but response factors derived from oxidative hydrolysates of standard proteins are more reliable in the range of 1 μg protein hydrolyzed (West and Crabb, 1992).

5. Always perform concurrent oxidative hydrolysis and analysis of control and unknown samples to verify the efficacy and accuracy of the analytical process.

   Incomplete compositional analysis results from oxidative hydrolysis—His, Met, Tyr, and Trp are also modified. Quantitation of these residues should be obtained in separate analyses using other hydrolysis conditions.

SUPPORT PROTOCOL 13

Pyridylethylation for Quantification of Cysteine

Alkylation of cysteine with 4-vinylpyridine produces pyridylethylcysteine, which is stable to acid hydrolysis (Friedman et al., 1970) and readily measured by PTC-AAA. Pyridylethylcysteine (Pec) is commercially available (Pierce) and can be used to determine an appropriate Pec response factor as described for other standard amino acids. The following protocol is adapted from Hawke and Yuan (1987).

Additional Materials (also see Basic Protocol)

1:3 (v/v) 1 M Tris·HCl (pH 8.5) containing 4 mM EDTA (Sigma)/8 M guanidine·HCl *or* 1 M Tris·HCl/1 mM EDTA, pH 8.5, containing 8 M urea (ultrapure)
1 M Tris·HCl, pH 8.5, or 1 N HCl, for adjusting pH if necessary

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10% (w/v) 2-mercaptoethanol
4-vinylpyridine (Aldrich)

Additional reagents and equipment for desalting sample (see Support Protocols for Sample Preparation)

1. Dry the sample and dissolve in <50 µl of either 1:3 (v/v) 1 M Tris·HCl, pH 8.5, containing 4 mM EDTA/8 M guanidine·HCl or 1 M Tris·HCl/1 mM EDTA, pH 8.5, containing 8 M urea. Check pH by spotting ~1 µl on pH paper; adjust pH to ~8.5 with 1 M Tris·HCl or 1 N HCl.

   Avoid guanidine·HCl if the sample contains SDS.

2. Add 2.5 µl freshly prepared 10% (w/v) 2-mercaptoethanol, flush with argon, seal, and incubate 2 hr at room temperature.

3. Add 2 µl 4-vinylpyridine neat, mix, and incubate 2 hr under argon.

   Always use fresh 4-vinylpyridine for pyridylethylation.

4. Desalt immediately by RP-HPLC, dialysis, solvent exchange (with a Microcon concentrator), or precipitation (see Support Protocols for Sample Preparation).

   Avoid SDS if possible; remove SDS by acetone precipitation.

5. Hydrolyze, PTC derivatize, and analyze for pyridylethylcysteine (see Fig. 11.9.1).

Hydrolysis with HCl/Dodecanethiol for Quantification of Tryptophan

SUPPORT PROTOCOL 14

The dodecanethiol/HCl hydrolysis method was the most successful method for Trp quantification in the comparative study of Strydom et al. (1993). The method involves automatic vapor-phase hydrolysis in the PE Applied Biosystems model 420H Amino Acid Analysis system.

***Additional Materials** (also see Basic Protocol)*

40% (w/v) dodecanethiol (Fluka) in heptane (Burdick and Jackson)
Standard protein containing tryptophan, e.g., cytochrome *c*, α-lactalbumin, or lysozyme
Amino Acid Analysis system (PE Applied Biosystems model 420H)

Additional reagents and equipment for determining response factors for difficult residues (see Support Protocol 11)

1. Apply 40 µl of 40% dodecanethiol (in heptane) to the sample frit of the PE Applied Biosystems model 420H Amino Acid Analysis system 10 to 15 min prior to sample application. Apply the sample and proceed with the normal autohydrolysis process.

   For further details, see Bozzini et al. (1991) and West and Crabb (1992).

2. Determine response factors for Trp by hydrolysis and PTC-AAA of standard Trp-containing proteins such as cytochrome *c* (1 Trp/mol), α-lactalbumin (4 Trp/mol), and lysozyme (6 Trp/mol; see Support Protocol 11).

   A Trp standard is useful for optimizing chromatography, but response factors derived from hydrolysates of standard proteins are more reliable in the range of 1 µg protein hydrolyzed (West and Crabb, 1992).

3. Perform concurrent dodecanethiol/HCl hydrolysis and analysis of control and unknown samples to verify the efficacy and accuracy of the analytical process.

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SUPPORT PROTOCOL 15

Hydrolysis with 4 N Methanesulfonic Acid for Quantification of Tryptophan

This alternative procedure, hydrolysis with 4 N methanesulfonic acid, is adapted from Hsi and Yamane (1994). Response factors for Trp are determined from 4 N methanesulfonic acid hydrolysates of standard proteins and concurrent hydrolysis and analysis of control and unknown samples.

Additional Materials (also see Basic Protocol)

4 N methanesulfonic acid
4 N NaOH

1. Add 30 µl of 4 N methanesulfonic acid to the dried sample in a clean 6 × 50–mm tube. Seal sample tubes in bulge-modified 40-ml screw-cap vials and alternately flush with argon and evacuate as described for routine vapor- and liquid-phase HCl hydrolysis (see Support Protocol 7 or 8). Seal under argon rather than vacuum.

   Batchwise hydrolyses are recommended.

2. Heat 1 hr at 165°C, then cool to room temperature. Add 30 µl of 4 N NaOH to neutralize, then vacuum dry.
3. PTC derivatize and analyze (see Basic Protocol, steps 8 to 14).

SUPPORT PROTOCOL 16

Quantification of pSer, pThr, and pTyr

Chromatographic resolution and measurement of PTC-phosphoamino acids are straight forward and relatively simple (see Fig, 11.9.8). However, the acid lability of the phosphate moiety makes hydrolysis the most critical part of the analytical process. Phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) are analyzed using a time course of liquid-phase HCl hydrolysis (see Support Protocol 8). Commercially prepared standards of the three phosphoamino acids are used to optimize chromatographic conditions and to determine response factors (see Support Protocol 11). Synthetic phosphopeptides can also be used to determine the response factors following limited HCl hydrolysis, but any such peptide must contain a well-defined amount of the phosphoamino acid of interest.

Additional Materials (also see Basic Protocol and Support Protocol 8)

Standard phosphoamino acids

Additional reagents and equipment for liquid-phase hydrolysis (see Support Protocol 8) and determining response factors for difficult residues (see Support Protocol 11)

1. Perform a time course liquid-phase HCl hydrolysis at 110°C for 1, 2, 3, and 4 hr (see Support Protocol 8). Vacuum dry the hydrolysate.
2. PTC derivatize and analyze (see Basic Protocol, steps 8 to 14). Establish the chromatography conditions needed for optimum resolution of PTC-phosphoamino acids on the HPLC system using standard phosphoamino acids.

   An example of PTC-AAA resolution of pSer, pThr, pTyr, and hydroxyproline is presented in Figure 11.9.8.

   CAUTION: *Extended use of silica-based RP-HPLC columns near neutral pH (as in Fig. 11.9.8) can shorten the useful lifetime of the column.*

3. Estimate response factors from analysis of standard phosphoamino acids and by hydrolysis and PTC-AAA of standard synthetic phosphopeptides of defined phosphate content (see Support Protocol 11).
4. Perform concurrent HCl hydrolysis and analysis of control and unknown samples to verify the efficacy and accuracy of the analytical process.

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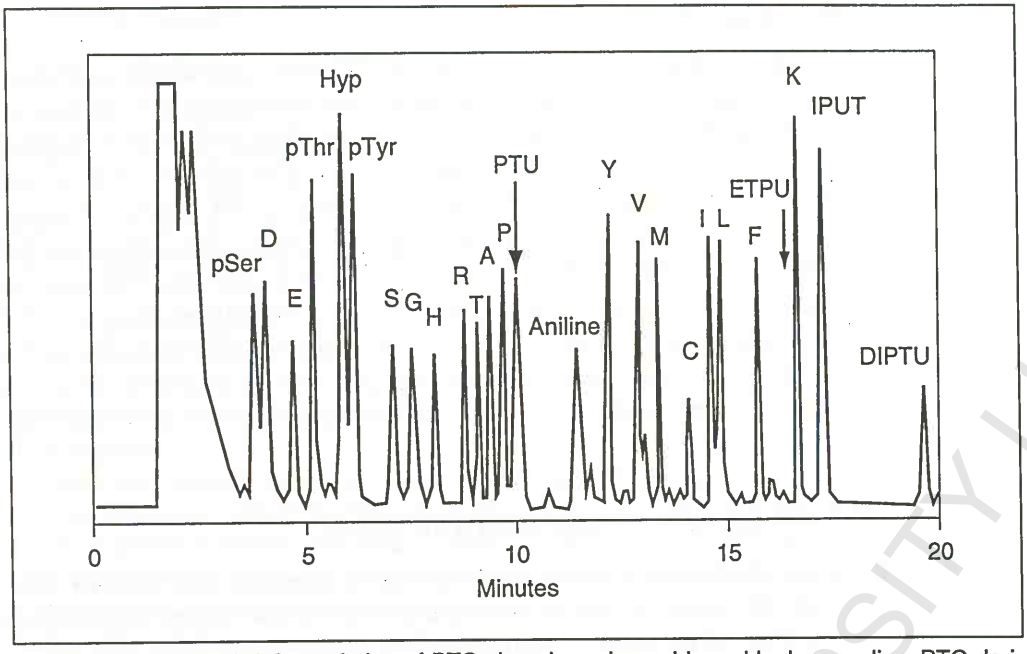

Figure 11.9.8 RP-HPLC resolution of PTC-phosphoamino acids and hydroxyproline. PTC-derivatized amino acid Standard H plus pSer, pThr, pTyr, and Hyp (120 pmol) were chromatographed using the PE Applied Biosystems 130 HPLC and PE Applied Biosystems PTC C_{18} column (2.1 × 220–mm) with a flow rate of 300 µl/min, column temperature of 36°C, and the following gradient: time 0, 7% B; time 4 min, 14% B; time 10 min, 34% B; time 20 min, 66% B; and time 25 min, 100% B. Solvent A was 120 mM sodium acetate, pH 6.45, and solvent B was 31.5 mM sodium acetate containing 70% acetonitrile. Amino acids are identified using the single-letter amino acid code (see Table A.1A.1). Abbreviations (also see Fig. 11.9.1): pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine; Hyp, hydroxyproline.

Quantification of pSer as PTC-*S*-Ethylcysteine

SUPPORT PROTOCOL 17

Phosphoserine (pSer) can be quantified by first converting it to *S*-ethylcysteine (see Meyer et al., 1991), then hydrolyzing and derivatizing it for PTC-AAA. A synthetic phosphopeptides containing a well-defined amount of pSer is used as a standard.

Additional Materials (also see Basic Protocol and Support Protocols 7 and 8)

Performic acid solution: 95:5 (v/v) performic acid (Sequenal grade)/hydrogen peroxide (30% by mass)
Modification mixture (see recipe)
Acetic acid
Standard synthetic phosphopeptides with defined phosphate concentration
Additional reagents and equipment for vapor-phase hydrolysis (see Support Protocol 7) or liquid-phase hydrolysis (see Support Protocol 8)

1. Dry sample in a 6 × 50–mm tube. Add 5 µl performic acid solution and incubate 1 hr on ice to oxidize cysteine to avoid false positives. Redry.
2. Add 20 µl modification mixture. Incubate 1 hr at 50°C under nitrogen. Acidify with 5 µl acetic acid and vacuum dry.
3. Perform standard vapor- or liquid-phase HCl hydrolysis (see Support Protocol 7 or 8).
4. PTC derivatize and analyze (see Basic Protocol, steps 8 to 14) in comparison with synthetic standard phosphopeptides.

pThr and pTyr do not form equivalent derivatives. O-Glycosylated Ser will also yield S-ethylcysteine in this procedure.

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SUPPORT PROTOCOL 18

Analysis for Hydroxyproline

Perform standard HCl hydrolysis (see Support Protocol 7 or 8) and PTC derivatization (see Basic Protocol). No special hydrolysis or modification methods are required to quantify hydroxyproline; however, PTC-hydroxyproline may coelute with other residues such as glutamic acid (Glu). Prepare a hydroxyproline amino acid standard (Pierce) and optimize its elution position in the HPLC system by changing the pH of the chromatography solvents, generally to a slightly more alkaline pH. For example, changing the solvent pH from 5.4 to ~5.6 with 5 N NaOH in the PE Applied Biosystems PTC analysis system provides adequate resolution of hydroxyproline from Glu without changes in the gradient conditions (Fig. 11.9.1). To resolve hydroxyproline from pThr and pTyr in the PE Applied Biosystems system, increase the ionic strength of chromatography solvent A from 50 to ~120 mM sodium acetate (Mallinkrodt) and the pH from 5.4 to ~6.5 (Fig. 11.9.8).

SUPPORT PROTOCOL 19

IDENTIFICATION OF PROTEINS FROM AAA DATA

Amino acid analysis in combination with computer search programs (see *UNITS 2.4 & 2.5*) provides a valuable and rapid method for the identification of known and homologous proteins. Two useful search programs readily accessible via the World Wide Web are

1. PROPSEARCH (http://www.EMBL-Heidelberg.de/aaa.html) and
2. ExPASy (http://expasy.hcuge.ch/ch2d/aacompi.html).

These programs are relatively tolerant to error and provide a very good chance of identifying proteins in the database. More information about PROPSEARCH can be found in Hobohm et al. (1994), about ExPASy in Wilkins et al. (1996), and about applications of the methods in resource facilities in Schegg et al. (1997).

1. Contact the Website for PROPSEARCH at http://www.EMBL-Heidelberg.de/aaa.htm or ExPASy at http://expasy.hcuge.ch/ch2d/aacompi.html. Enter compositional mole % data (see Basic Protocol, step 34) on the Internet forms; also enter optional information about molecular weight and isoelectric point (pI) if available.
2. Execute both search programs and compare results.
3. Evaluate the computer hit lists.

   Smaller scores are better for both programs. For PROPSEARCH, scores <1.5 are worthy of attention, scores between 1.5 and 2.5 generally indicate less reliable identification, and scores >2.5 indicate unreliable identification. For ExPASy, scores of 0 to 25 are worthy of attention, scores of 25 to 50 provide less reliable identification, and scores >50 indicate unreliable identifications. (For examples, see Anticipated Results and Figs. 11.9.9 and 11.9.13.)

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Modification mixture

80 µl ethanol
65 µl 5 N NaOH
60 µl ethanethiol
400 µl H_2O, added last

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COMMENTARY

Background Information

This unit is meant primarily for readers with little or no experience in amino acid analysis (AAA) and focuses on the PTC method because (1) it is an established and reliable method of high-sensitivity AAA, (2) it is the most widely used method for picomole-level AAA, and (3) it is easier to find advice and knowledgeable assistance regarding the PTC method than any other method of high-sensitivity AAA.

Distinguishing characteristics of amino acid analysis

AAA is deceptively simple in concept, but it is one of the most difficult analytical methods in all of protein chemistry. The greatest value and benefit of the analysis is its quantitative capability, but measuring many different amino acids with quantitative accuracy is precisely what makes the analysis so difficult. AAA is a statistical type of measurement; peptide bonds and amino acids vary widely in their stability/lability to acid hydrolysis, oxidation, and modification. As noted previously, Trp and Cys are destroyed by HCl hydrolysis, Ser and Thr are partially destroyed, Ile and Val are slow to cleave, Met is subject to oxidation, and Asn and Gln are completely deamidated. Variable but ubiquitous background contamination plus variable instrument and analyst performance contribute to the statistical nature of the measurement and can confound the analysis, particularly at and below the low picomole level. Nevertheless, AAA remains a powerful tool. The technology offers the best way to quantify peptides and proteins in many biochemical studies, and it provides invaluable support to primary structure studies. It also provides quantitative compositional characterizations useful in verification of synthetic and recombinant polypeptide structures, identification of odd or modified residues, and identification of proteins by way of computer database searches.

Overview of AAA methods

Several methods of quantitative AAA exist, but all essentially involve either postcolumn derivatization following ion-exchange chromatography or precolumn derivatization followed by reversed-phase HPLC. The classical AAA methodology involves ion-exchange chromatography and postcolumn continuous reaction with ninhydrin (Moore and Stein, 1949; Moore et al., 1958). Moore and Stein were awarded the 1972 Nobel Prize in Chemistry for development of quantitative AAA technology. Advantages of the classical method include excellent instrumentation, valuable technical support from instrument vendors, and the ability to analyze samples in the presence of salt and other contaminants. In fact, protein in SDS-PAGE bands may be quantified using ion-exchange amino acid analyzers following HCl hydrolysis of the polyacrylamide gel band (Stein and Brink, 1981a; Williams and Stone, 1995). The primary disadvantage of the ion-exchange postcolumn ninhydrin method is that its sensitivity is limited to the high picomole/low nanomole range, which means that >2 μg of sample per analysis is usually required to obtain useful information (typically, 5 to 10 μg per analysis is required). However, fluorescence detection in place of ninhydrin enhances the sensitivity of the classical methodology to the low picomole range by using reagents such as fluorescamine or *o*-phthalaldehyde (OPA). A disadvantage of fluorescamine and OPA fluorescence–detection, postcolumn ion-exchange amino acid analyzers is that proline, a secondary amine, is not detected without an additional and relatively intricate oxidation step to convert the residue to a detectable primary amine (Stein and Brink, 1981b).

A number of precolumn-derivatization reversed-phase HPLC methods for AAA have evolved that provide useful and more sensitive alternatives to classical AAA. The precolumn methods are distinguished from one another by different derivatization chemistry and are more sensitive and generally less expensive to set up than the ninhydrin postcolumn method. Common precolumn derivatizing reagents for AAA include phenylisothiocyanate (PITC; Koop et al., 1982; Heinrikson and Meredith, 1984; Tarr, 1986), dimethylaminoazobenzenesulfonyl chloride (DABSYL; Knecht and Chang, 1986), OPA (Jones and Gilligan, 1983), 9-fluorenylmethyl chloroformate (Fmoc; Bank et al., 1996), OPA plus Fmoc (Blankenship et al., 1989), and 6-aminoquinnolyl-*N*-hydroxysuccinyl carbamate (AQC; Strydom and Cohen, 1994). Excellent instrumentation and valuable technical support are also available from instrument vendors for many of the precolumn approaches. Disadvantages of precolumn methods include sensitivity to derivatization interference from salt and other contaminants, multiple reaction by-products that may obscure chromatographic resolution of the amino acids, and variable stability of the derivatives. How-

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ever, effective ways of avoiding, minimizing, or eliminating these complications have been established for most of the precolumn methods.

Performance comparisons of the various precolumn and postcolumn AAA methods and documentation of the instrumentation used in studies spanning almost a decade and involving up to 77 different resource laboratories can be found in the annual AAA studies of the Association of Biomolecular Resource Facilities (ABRF; Niece et al., 1989; Crabb et al., 1990; Tarr et al., 1991; Strydom et al., 1992, 1993; Yücksel et al., 1994, 1995; Mahrenholz et al., 1996; Schegg et al., 1997). From 1989 through 1996, ~38% of the participants in these studies used the postcolumn ninhydrin methodology, ~48% used precolumn PTC methods, and 14% used other AAA methods. Overall these studies demonstrate that more than one method of AAA works well. It is important to recognize that the skill and experience of the analyst are probably the most important factors for successful AAA.

Costs of PTC-AAA

Excluding the investment in instrumentation, labor is the largest expense in PTC-AAA. Labor costs may be estimated on a per analysis basis by multiplying the hourly wage of the analyst by 1 to 2. Supply costs are modest and typically account for ~25% of the per analysis cost. For heavy-use AAA systems, a service contract from the instrument vendor may be worthwhile; the service contract can cost up to ~$8000 per year. A gradient HPLC system with at least a simple integrator is essential ($25,000 to $35,000 in 1996); add another ~$10,000 for an automatic sample injector. In 1996, the list price of a dedicated PTC-AAA system ranged from $50,000 to $80,000 depending on options and vendor.

Where to find help

Any biomolecular resource facility that routinely performs AAA is an excellent source of general information about AAA and also may be of assistance in troubleshooting. The ABRF sponsors an Amino Acid Analysis Research Committee that can help answer many questions concerning how to perform amino acid analysis. In addition, the ABRF publishes a Yellow Pages listing of Resource Facilities that will perform AAA and/or other analytical and synthetic services for out-of-house clients, generally for a fee. The ABRF Yellow Pages and Amino Acid Analysis Research Committee membership are accessible via the World Wide Web at the ABRF homepage (http://www.medstv.unimelb.edu.au/abrf.html) or by contacting the ABRF Business Office, FASEB, 9650 Rockville Pike, Bethesda, MD 20814-3998. As previously noted, the service and technical support offered by instrument companies selling amino acid analyzers can also be useful.

Critical Parameters

Performance overview

Essentially all low-picomole level AAA methods are labor-intensive, high-maintenance processes subject to a variety of possible complications. High-quality PTC-AAA performance should be expected but not without costs in time and energy. Vigilant effort is required to verify that the PTC-AAA system is functioning in a quantitatively reliable and trustworthy fashion. At least three analyses of an amino acid standard (e.g., Pierce Standard H) should be performed every day the system is used to ensure accurate calibration. In addition, daily analysis of a peptide or protein standard of known concentration is recommended to demonstrate that the performance provides accurate quantitation and low compositional error. Analysis of blank samples (e.g., solvent and reagent aliquots, empty PVDF pieces, and empty hydrolysis tubes) should be performed regularly and as needed to quantify background levels of amino acids and "junk" peaks in the chromatography. If the analysis system sits idle for a few weeks, expect to spend several days to a week reestablishing reliable performance levels. To maintain a reliably accurate high-sensitivity analysis system, expect that half of the overall analyses performed on the system may involve standards, blanks, and various tests (e.g., to establish Trp or Cys response factors), especially if the demand for experimental sample analysis is modest.

Maintenance

Instrument and system maintenance is a key factor in achieving successful performance from PTC-AAA. A systematic schedule for instrument maintenance should be established and all maintenance activities documented. Good record keeping practices will facilitate troubleshooting efforts when performance problems occur. Maintenance activity will vary from system to system, but daily maintenance activities should include preparing new derivatization reagents (for manual derivatization), cleaning the vacuum system cold trap, and checking for vacuum system leaks, HPLC de-

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tector lamp stability, HPLC leaks, and the quality of HPLC resolution of the amino acids. Weekly maintenance activities should include changing chromatography buffers and derivatization reagents, performing available self-tests on automatic instrumentation, cleaning away dust on and around the instrumentation, changing the vacuum pump oil, and cleaning the Speedvac evaporator. Monthly maintenance activities should include changing in-line filters and fan filters on the HPLC and/or automatic derivatizer. Periodically, HPLC pump seals (about twice a year), the UV detector lamp (1 to 2 per year), and HPLC columns (2 to 4 per year) must be replaced. Automatic micropipettors must also be calibrated and maintained in clean condition to achieve accurate quantitation in amino acid analysis.

Contamination

Background contamination plagues all high-sensitivity AAA procedures, not just PTC-AAA, and it seems to come from everywhere when attempting to perform measurements at the low-picomole level. Dust in the air can be one of the most difficult sources to deal with; dust can contribute high levels of glycine, serine, and alanine to the results. Keep the laboratory room as clean and tidy as possible. Locate the AAA workstation and instrumentation in a low-traffic area, and avoid placing the work area directly under heater and air conditioner vents.

All reagents and solvents can be sources of contaminants (e.g., water, HCl, derivatizing chemicals, and sample preparation buffers). Use the highest purity solvents and reagents available. To minimize UV-absorbing phenylthiourea by-products, avoid ammonia-based solvents. Be aware of the age of the solvents and reagents purchased and use the newest lots available. Date the chemicals as they are received in the laboratory to assist with troubleshooting when problems arise. During the analysis, keep solvents and reagents in covered amber bottles away from sunlight. Store phenylisothiocyanate (PITC), diisopropylethylamine (DIEA), triethylamine (TEA) amino acid Standard H, and peptide/protein standards at –20°C under argon or nitrogen. Monitor the chromatography solvents for "floaters"; bacteria grow well in sodium acetate buffers, and sunlight enhances their growth.

Meticulous sample handling technique is also essential for high-quality analyses. Glassware and other lab ware that comes in contact with the sample or is used in preparation of reagents and chemicals for hydrolysis, derivatization, and analysis are other sources of contaminants and should always be cleaned before use. Keep micropipet tips and 6 × 50–mm tubes in a dust-free, covered box. Avoid touching pipet tips, avoid unnecessary opening and closing of sample vials, and wear powder-free gloves when handling samples. A more detailed description of sources of contamination and ways to reduce background in PTC-AAA can be found in Atherton (1989).

Troubleshooting

Table 11.9.1 is a brief, generic troubleshooting guide for common problems encountered in PTC-AAA; it is not meant to be all-inclusive. The analyst should query instrument companies providing support for PTC-AAA about the availability of more detailed troubleshooting guides for PTC-AAA on their particular instrumentation.

Anticipated Results

Average PTC-AAA data

The quality of AAA results is significantly influenced by the amount of sample hydrolyzed, as shown in the PTC-AAA results from pyridylethyl ribonuclease (see Table 11.9.2). These data represent average results from two to three hydrolyses and illustrate that compositional error and variability increase as the amount of protein hydrolyzed decreases. Lower recovery is also evident from the smaller amounts of sample hydrolyzed. With clean sample preparations and careful technique, an average compositional error of ≤10% can be obtained with 0.5 to 1 µg of sample hydrolyzed, with the best analyses showing 0% to 5% error from a single hydrolysis/analysis. Data exhibiting relatively high compositional error can still provide useful estimates of the quantity of protein adequate for many applications (e.g., estimating whether there is enough sample to justify proteolysis and subsequent peptide purification and sequence analysis). Furthermore, the results from 0.2 µg of hydrolysate in Table 11.9.2 allow correct identification of the sample via the PROPSEARCH and ExPASy computer database queries (Fig. 11.9.9), despite 21% average compositional error.

Realistic expectations for the quality of AAA results can be obtained from reviewing the published AAA studies from the ABRF (Niece et al., 1989; Crabb et al., 1990; Tarr et al., 1991; Strydom et al., 1992, 1993; Yücksel

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Table 11.9.1 Troubleshooting Guide for PTC Amino Acid Analysis[a]

Problem	Possible cause	Possible solution
No peaks in HPLC chromatogram	Sample is not injected	Unclog or replace tubing; purge autosampler lines
	UV lamp is not working	Check that the lamp is on; replace the lamp
No peaks on chromatogram but injection front present	Sample not being derivatized	Check/replace derivatizing reagents; replace lines or bottle seals; replace old reagents
No amino acid peaks detected but background "junk peaks" are present	No hydrolysis	Make certain hydrolysis vessel has a proper seal; make certain proper vacuum or inert gas is present
	Not enough protein/peptide in sample	Try with ten times more sample
Poor peak size reproducibility and irregular recovery	Pipetting errors	Recalibrate pipettors
	Incomplete hydrolysis	Improper seal on hydrolysis vessel; replace slide valve
	Incomplete derivatization	Check deliveries of derivatizing reagents; replace lines, bottle seals, or reagents
	Too much salt or detergent in sample	Desalt sample by RP-HPLC, microdialysis, Centricon, or precipitation
	Improper sample injection	Unclog or replace tubing
	UV lamp getting old	Replace UV lamp
Inaccurate quantitation of standard peptides and proteins	Erroneous calibration file	Recalibrate
	Incorrect concentration used for amino acids and/or protein standards	Replace standards
	Incomplete hydrolysis and/or derivatization	Repeat hydrolysis and derivatization with new sample
Low Asp and Glu	Metal contamination from glass hydrolysis tubes	Prior to hydrolysis, dry samples in 6 × 50–mm tubes from 0.25 µg/µl EDTA
High amino acid background in control blanks	Contamination in solvents, reagents, glassware, micropipets, etc.	Change reagents/utensils; use more care during hydrolysis/derivatization
	Dust (= Gly, Ser, and Ala)	Clean workstation and instrument
	Hands and fingers	Wear powder-free gloves
	Dirty column	Clean or replace column
High by-product (PTU, IPTU, aniline, etc.) and "junk" peaks in control blanks	Old derivatization reagents	Replace PITC, DIEA, etc.
	Inadequate drying step	Vacuum dry longer
Unusually large and unidentifiable chromatography peaks	Air injected with sample	Completely fill (overfill) the sample loop with liquid; clean/replace clogged delivery lines; degas solvents; maintain constant solvent temperature
	Air bubble in flow cell	Install narrower tubing and/or back pressure device on flow cell outlet

continued

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Table 11.9.1 Troubleshooting Guide for PTC Amino Acid Analysis[a], continued

Problem	Possible cause	Possible solution
Poor retention time reproducibility	Improper equilibration and/or gradient cycle	Extend equilibration time and/or redesign gradient
	Leaky HPLC system	Tighten or replace leaky fittings, pump seals, tubing, column, and flow cell connections
	Inconsistent column temperature	Control column temperature electronically
	Faulty gradient or flow rate control	Repair or replace microprocessor controller and mixing chamber
Poor peak shape	Improper pH and/or composition of solvents and/or transfer buffer	Reevaluate buffer recipe, change formula as needed, and remake buffers
	HPLC column too old	Replace column
	Injection volume too large	Decrease injection volume
Noisy HPLC baseline	Old UV lamp	Replace lamp
	Improper flow or mixing	Repair/replace leaks, microprocessor, and mixer
	Poor electrical connections	Tighten/replace electrical connections between detector and integrator
	Dirty/old column	Replace column

[a]Abbreviations: DIEA, diisopropylethylamine; PITC, phenylisothiocyanate

et al., 1994, 1995; Mahrenholz et al., 1996; Schegg et al., 1997). These reports provide an overview of the accuracy obtained by all participants of the study itemized by method (e.g., postcolumn ninhydrin versus precolumn PTC, OPA/Fmoc, AQC) and usually highlight the best results from both precolumn and postcolumn methods.

Reproducibility and calibration data

The average peak areas (or peak heights) and retention times for standard amino acids vary with derivatization conditions (e.g., reagent concentration and age), chromatography conditions (e.g., injection reproducibility, solvent, column age, and temperature), pipetting accuracy, and background contamination. The AAA system reproducibility must be routinely evaluated. An example of relevant data for this purpose using two of the sixteen standard amino acids is shown in Table 11.9.3. This type of statistical evaluation should be performed for all sixteen amino acids used for calibration and summarized with average relative standard deviation (RSD) values as shown in Table 11.9.4. The reproducibility of the system must be maintained such that average RSD values for all sixteen amino acids do not exceed an upper limit, for example, 8% RSD for peak area and 0.4% RSD for retention time. RSD values typically increase with smaller sample amounts (see Table 11.9.2), indicating less reproducibility and more variability. Once the average peak area (or average peak height) has been satisfactorily determined for all the amino acids, response factors may be calculated by dividing the average areas by the picomoles analyzed. A calibration file should be compiled as illustrated in Table 11.9.5. The calibration file should include a retention time window which sets retention time limits for the integrator to identify and quantify the various peaks (e.g., ±0.15 min in Table 11.9.5).

Sensitivity range

Amino acids must be measured in the linear response range of the analysis system to obtain meaningful results. The appropriate amount of sample should allow quantification of both the low-abundancy and high-abundancy residues in a given peptide or protein. However, in practice, the results sometimes fall outside the meaningful response range because too much or too little sample was analyzed. The analyst must learn to recognize unreliable data beyond the upper limit of the range as well as be cognizant of the influences of background level contamination and hydrolysis losses in the lower end. An example of PTC-AAA data demonstrating a linear response between the

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amount analyzed and peak area is shown for the sixteen standard amino acids in Figure 11.9.10. For this particular analysis system, the response of most residues plateaus above 5 nmol; lysine, which has two PITC-reactive amino groups, plateaus at a lower level (~4 nmol).

Another useful quantitative evaluation of every PTC-AAA system is a comparison of the amount of sample recovered following HCl hydrolysis. Average recovery data from 123 manual vapor-phase HCl hydrolyses of nine different proteins performed between ~65 and 2600 ng are shown in Figure 11.9.11 (West and Crabb, 1990). These data show that essentially quantitative recovery was obtained from amounts hydrolyzed in 6 × 50–mm tubes in the 400 to 2600–ng range but that losses occurred for amounts hydrolyzed at or below ~230 ng. Average data from 336 automatic vapor-phase HCl hydrolyses of the same nine proteins showed markedly better recovery from automatic than manual hydrolyses below the 400-ng level and a concomitant improvement in compositional accuracy (West and Crabb, 1990). For the majority of analysts without automatic instrumentation, the sensitivity of PTC-AAA is not limited by the detection chemistry or current chromatography capabilities but rather by complications introduced by hydrolysis and background contamination. Sample amounts of 0.5 to 2 μg usually provide reasonable results for routine PTC-AAA following standard HCl hydrolysis conditions.

Table 11.9.2 Average PTC AAA Results from Pyridylethyl Ribonuclease[a,b]

Amino acid	Known composition[c]	Amount hydrolyzed (μg)				
		0.06	0.2	0.4	1.0	2.6
		Residues per molecule				
Asx	15	18.3	16.8	17.2	16.0	16.6
Glx	12	11.7	12.3	13.2	12.7	12.7
Ser	15	16.2	14.9	15.3	12.5	13.4
Gly	3	8.6	5.3	4.3	3.3	3.4
His	4	3.3	3.5	4.3	3.2	3.1
Arg	4	5.9	5.2	5.2	5.2	5.1
Thr	10	8.8	9.6	10.0	9.6	10.2
Ala	12	11.1	12.6	13.5	12.6	12.6
Pro	4	2.3	3.6	3.9	3.7	3.7
Tyr	6	10.9	7.3	6.5	6.6	6.5
Val	9	5.9	8.5	8.5	9.1	8.8
Met	4	0.8	1.3	2.0	2.1	3.7
Ile	3	0.6	1.5	1.5	2.1	1.8
Leu	2	2.0	2.2	2.1	2.2	2.2
Phe	3	1.7	2.6	2.9	3.3	3.3
Lys	10	12.0	10.4	11.5	11.7	7.6
Pec	8	6.2	8.3	9.7	8.3	9.1
pmol analyzed		2.7	10	19	66	189
pmol hydrolyzed		4.0	12	24	71	192
Number of analyses		2	2	3	3	3
Average peak area RSD		15.7	13.2	3.7	2.8	2.2
Average % error		34	21	17	11	9
Average % recovery		67	82	78	108	96

[a]Manual vapor-phase HCl hydrolysis was performed at 150°C for 1 hr. Automatic PTC derivatization and analysis were performed with PE Applied Biosystems models 420H, 130, and 920 instrumentation as described in West and Crabb (1990).

[b]Abbreviations: Pec, pyridylethyl cysteine; RSD, % standard deviation or relative standard deviation. Amino acids are identified using the three-letter abbreviations (see Table A.1A.1).

[c]Relative standard deviation (standard deviation of the mean/mean) × 100.

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Results from PROPSEARCH (http://www.embl-heidelberg.de/aaa.html)

Rank	ID	DIST	LEN2	POS1	POS2	pI	DE
1	rnp_antam	1.89	124	1	124	8.43	Ribonuclease Pancreatic
2	rnp_girca	1.95	124	1	124	7.22	Ribonuclease Pancreatic
3	rnp_conta	2.12	124	1	124	8.24	Ribonuclease Pancreatic
4	rnp_sheep	2.14	124	1	124	7.83	Ribonuclease Pancreatic
5	rnp_bubar	2.29	124	1	124	7.83	Ribonuclease Pancreatic
6	rnp_gazth	2.30	121	1	121	8.43	Ribonuclease Pancreatic
7	rnp_aepme	2.32	124	1	124	8.43	Ribonuclease Pancreatic
8	rnp_hipam	2.62	124	1	124	7.59	Ribonuclease Pancreatic
9	rnp_macru	2.65	122	1	122	8.06	Ribonuclease Pancreatic
10	>p1;s44712	2.65	192	1	192	10.22	Opacity Protein-Neisseria

Results from ExPASy (http://expasy.hcuge.ch/ch2d/aacompi.html))

Rank	Score	Protein	pI	Mw	Description
1	28	RNP_BOVIN	8.64	13690	Ribonuclease Pancreatic
2	36	RNP_CONTA	8.64	13686	Ribonuclease Pancreatic
3	37	RNP_DAMKO	9.60	13709	Ribonuclease Pancreatic
4	38	POLG_POL1M	8.25	7385	Coat Protein VP4
5	38	POLH_POL1M	8.25	7385	Coat Protein VP4
6	39	POLG_POL32	8.20	7313	Coat Protein VP4
7	39	POLG_POL3L	8.20	7313	Coat Protein VP4
8	40	POLG_POL2L	8.25	7339	Coat Protein VP4 (P1A)
9	40	POLG_POL2W	8.25	7339	Coat Protein VP4 (P1A)
10	40	RNP_TAUOR	9.30	13742	Ribonuclease Pancreatic

Figure 11.9.9 Identification of RNase from PTC-AAA data. The PTC-AAA data in Table 11.9.2 from hydrolysis of 12 pmol (~0.2 µg) ribonuclease (RNase) was converted to mole % and entered into the PROPSEARCH and ExPASy database query programs for identification of the protein. The following mole % data were entered: Arg 4.42, Met 1.11, Glx 10.46, Ile 1.28, Ser 12.67, Leu 1.87, His 2.98, Phe 2.21, Gly 4.51, Lys 8.84, Thr 8.16, Asx 14.29, Ala 10.71, Pro 3.06, Tyr 6.21, Val 7.23, sum 99.81. The top ten scores from both programs are displayed. Despite the 21% compositional error in the AAA data, both programs correctly identified the protein. Abbreviations for the PROPSEARCH results: DIST, score; LEN2, length of sequence; POS1 and POS2, begin and end of the sequence; pI, isoelectric pH; DE, description. PROPSEARCH identification scores <1.5 are best, 1.5 to 2.5 are less reliable, and scores >2.5 are unreliable; for ExPASy, scores 0 to 25 are best, 25 to 50 less reliable, and >50 unreliable.

Using raw AAA data to identify a protein

Once the AAA system is calibrated and the precision and linear response range of the system are established, experimental results can be generated as shown in Figure 11.9.12. Raw data from PTC-AAA of a 36-kDa protein are presented; the picomoles of amino acids have been calculated from peak area using a calibration file stored in the integrator (Fig. 11.9.12). Before proceeding to any other calculation, the analyst should verify that the amino acid peaks have been correctly identified. The amino acid composition of the protein may then be calculated from the picomole values as described and exemplified in Figures 11.9.2 through 11.9.7. The easiest amino acid compositional calculation is mole %, and this is the format that is normally required to identify the protein by computer query of composition databases. Entering the mole % data calculated from Figure 11.9.12 into the PROPSEARCH and ExPASy database query programs provides correct identification of the protein as the cellular retinaldehyde-binding protein (CRALBP; Fig. 11.9.13). Ambiguity exists regarding the species in this identification (bovine and human CRALBP are 91% identical); however, the source of most AAA samples is known, and this does not constitute a major problem.

The raw data in Figure 11.9.12 were generated from hydrolyzing ~1.9 µg of protein and analyzing 50% of the hydrolysate. The data exhibit ~6.9% average compositional error using the calculation method described in Figure

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Table 11.9.3 Amino Acid Analysis Reproducibility and Calibration Data[a]

Sample ID	Retention time (min)	Peak height	pmol by height	Peak area	pmol by area
Peak ID: Alanine					
31102001	9.60	45424	210.1	197668	260.1
31102002	9.65	45252	209.3	197040	259.3
31102003	9.67	43070	199.2	190578	250.8
31202001	9.62	43397	200.7	192789	286.3
31202002	9.67	46658	215.8	203824	302.7
Minimum	9.60	43070	199.2	190578	250.8
Maximum	9.67	46658	215.8	203824	302.7
Average	9.64	44760	207.0	196380	271.8
Std dev	0.03	1500	6.9	5101	21.8
RSD	0.31	3	3.4	3	8.0
Peak ID: Arginine					
31102001	8.82	46723	210.5	199489	222.5
31102002	8.87	47794	215.3	207920	231.9
31102003	8.88	48632	219.1	210325	234.5
31202001	8.83	44862	202.1	195017	243.8
31202002	8.88	49562	223.3	211775	264.8
Minimum	8.82	44862	202.1	195017	222.5
Maximum	8.88	49562	223.3	211775	264.8
Average	8.86	47515	214.0	204905	239.5
Std dev	0.03	1815	8.2	7293	16.8
RSD	0.34	4	3.8	4	6.7

[a]Amino acid Standard H (300 pmol, Pierce) was analyzed five times; results from two of the sixteen amino acids are shown before instrument calibration. Std dev, standard deviation of the mean; RSD, % standard deviation of the mean or relative standard deviation. Automatic PTC derivatization, analysis, and statistical calculations were performed with PE Applied Biosystems models 420H, 130, and 920 instrumentation.

11.9.6. Note that the PROPSEARCH and ExPASy scores for CRALBP in Figure 11.9.13 are much lower and in a more reliable range than those for RNase in Figure 11.9.9, where the PTC-AAA data were derived from a smaller amount of sample (0.2 µg hydrolyzed) and exhibited greater error (21%). Despite ambiguities and a margin for error, computer searches of amino acid composition databases offer a valuable tool for the identification of proteins from minute amounts of sample. This relatively new application of amino acid analysis will likely become more important and widespread as more compositional data become available from gene sequencing.

Time Considerations

Amino acid analysis is a time-consuming process, and new practitioners should anticipate several weeks to initially establish a system in addition to ongoing effort to maintain the system performance in a trustworthy fashion. The following time allocations for the various steps in PTC-AAA are reasonable after developing some experience with the methodology.

Sample preparation is the most variable and potentially the longest step. Assuming purification has been accomplished, desalting and/or sample concentration can usually be achieved within a few hours.

The standard HCl hydrolysis step requires an hour for the vapor-phase method and 20 to 24 hr for the liquid-phase method; microwave vapor-phase HCl hydrolysis requires 20 min or less. Many samples can be hydrolyzed at once by any of these methods; allow ~1 hr total for handling 20 samples before and after heating (e.g., setting up the hydrolysis and drying the hydrolysates).

The manual PTC derivatization reaction requires a 20-min incubation; allow ~30 min for handling 20 samples (e.g., setting up the reaction and drying the final product).

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Table 11.9.4 Summary of Reproducibility and Calibration Data[a]

Peak ID	Number of peaks	Average retention time (min)	RSD	Average peak height	RSD	Average peak area	RSD
Asx	5	5.81	0.51	61112	2.95	253139	9.20
Glx	5	6.16	0.41	46318	2.67	201011	7.34
Ser	5	7.59	0.40	39202	3.17	177504	6.23
Gly	5	7.99	0.38	35917	2.88	163656	7.45
His	5	8.26	0.37	40133	2.62	177585	6.94
Arg	5	8.86	0.34	47515	3.82	204905	6.70
Thr	5	9.31	0.33	44320	2.87	192943	5.57
Ala	5	9.64	0.31	44760	3.35	196380	8.03
Pro	5	9.88	0.27	29321	2.52	127821	7.05
Tyr	5	12.40	0.19	60110	3.91	263636	7.74
Val	5	13.35	0.21	50073	3.29	220594	6.85
Met	5	13.78	0.20	49079	7.28	216244	7.83
Ile	5	15.49	0.16	51292	3.72	229503	7.42
Leu	5	15.72	0.19	48384	4.06	211669	7.10
Phe	5	16.56	0.18	53671	3.59	234958	7.04
Lys	5	17.69	0.17	80504	4.98	357280	6.69
Average RSD			0.29		3.61		7.20

[a]Summary of average retention times, peak heights, peak areas, and relative standard deviation (RSD) values (reproducibility) from five PTC analyses of 300 pmol of amino acid Standard H. Response factors are calculated by dividing average peak heights and average peak areas by picomoles of amino acid analyzed (300 pmol in this case).

Table 11.9.5 Amino Acid Standard Calibration File[a]

Peak ID	Retention time (min)	Retention time window (min)	Response factors	
			Height per pmol	Area per pmol
Asx	5.8	0.15	203.7	843.8
Glx	6.2	0.15	154.4	670.0
Ser	7.6	0.15	130.7	591.7
Gly	8.0	0.15	119.7	545.5
His	8.3	0.15	133.8	592.0
Arg	8.9	0.15	158.4	683.0
Thr	9.3	0.15	147.7	643.1
Ala	9.7	0.15	149.2	654.6
Pro	9.9	0.15	97.7	426.1
Tyr	12.4	0.15	200.4	878.8
Val	13.4	0.15	166.9	735.3
Met	13.8	0.15	163.6	720.8
Ile	15.5	0.15	171.0	765.0
Leu	15.7	0.15	161.3	705.6
Phe	16.6	0.15	178.9	783.2
Lys	17.7	0.15	268.4	1190.9

[a]Response factors were calculated from the average peak heights and peak areas listed in Table 11.9.4. The retention time window restricts amino acid peak identification to a defined time span, in this case ±0.15 min about each retention time.

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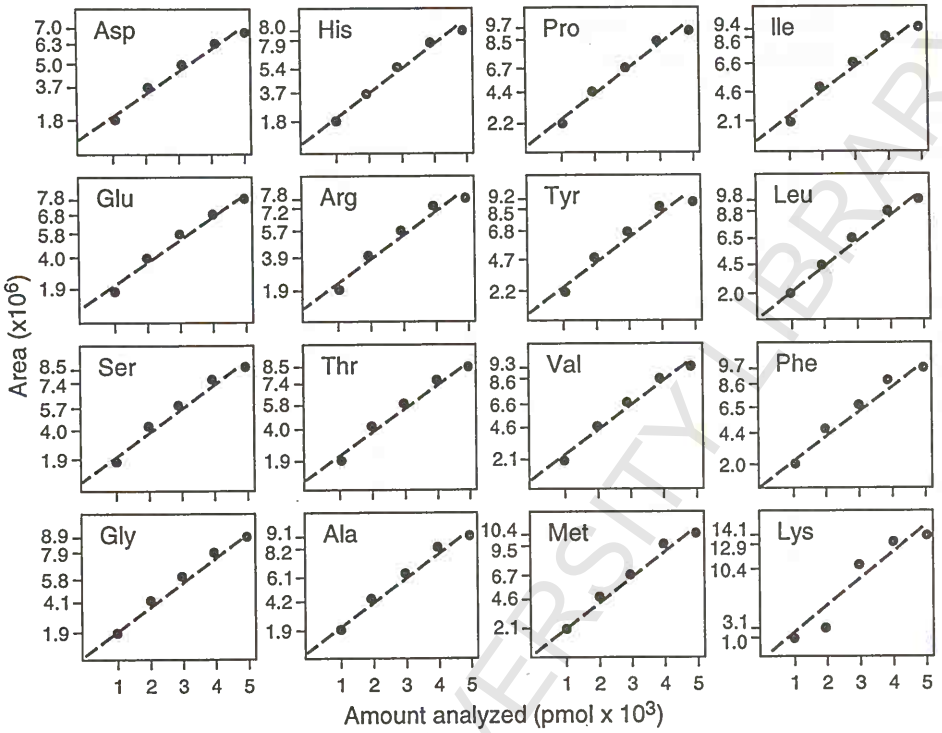

Figure 11.9.10 Linear response range for sixteen PTC amino acids. PTC amino acid Standard H (Pierce) was analyzed in replicate using PE Applied Biosystems models 420H, 130, and 920 instrumentation and PTC-AAA column (2.1 × 220–mm). A linear response of peak area versus amount analyzed was obtained between 10 pmol and 5 nmol for most residues (data <1 nmol not shown).

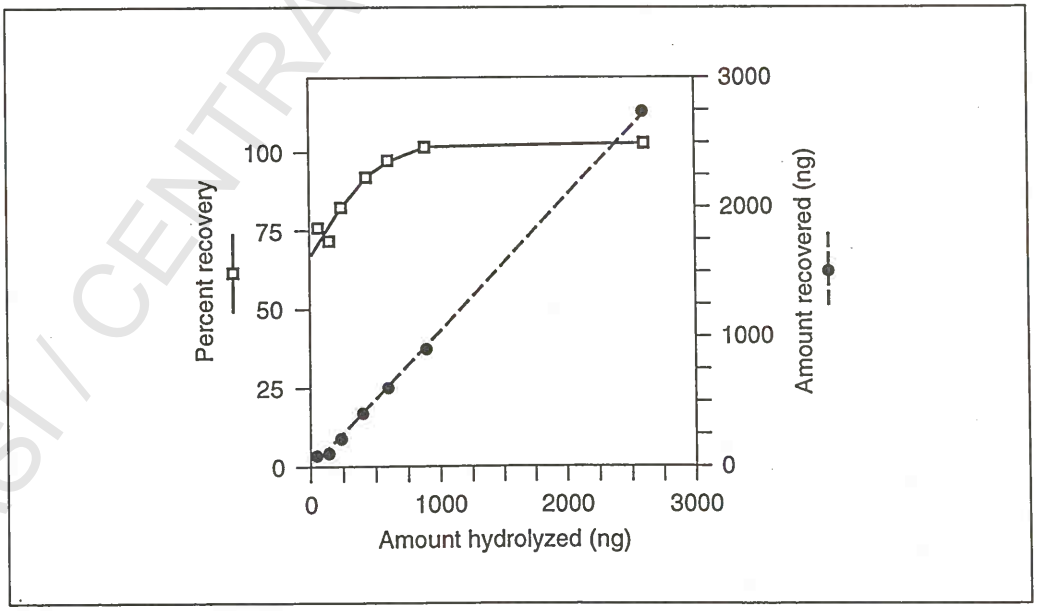

Figure 11.9.11 PTC-AAA linear response range following HCl hydrolysis. Nine different proteins were subjected to manual vapor-phase HCl hydrolysis in replicate and 123 analyses performed using PE Applied Biosystems PTC-AAA models 420H, 130, and 920 instrumentation. Average amounts hydrolyzed (in nanograms) are plotted versus both the average amounts recovered (in nanograms) and the percent recovery. These results demonstrate a linear response from ~400 to 2600 ng hydrolyzed but also show that losses occur for hydrolyses ≤230 ng (West and Crabb, 1990).

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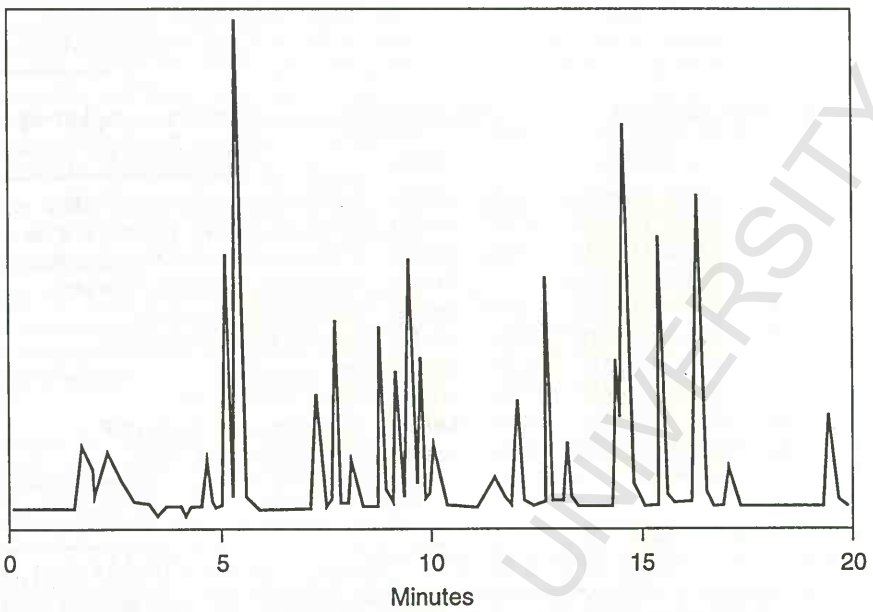
Time requirements vary for the chromatography step depending on the HPLC system but typically involve 30 to 60 min from injection to injection (e.g., 15 to 30 min for separation followed by another 15 to 30 min to clean and reequilibrate the column). Allow ~30 min to dissolve 20 samples and to load or program an autosampler. If no autosampler is available, allow all day for manual injections. Automatic derivatization and analysis with or without automatic hydrolysis generates about one analysis per hour with significantly reduced time required for sample handling.

Once the calibration process has become routine, plan on ~30 to 45 min to check and adjust retention time windows, reprocess data,



Peak ID	Retention time (min)	Peak area	pmol by area
Asx	5.05	3250442	1162.44
Glx	5.32	6767374	2729.84
Ser	7.15	1235546	728.13
Gly	7.59	1952006	1175.61
His	7.99	451547	271.38
Arg	8.68	2097680	1114.78
Thr	9.08	1576640	763.55
Ala	9.40	2837580	1294.95
Pro	9.67	1668884	680.52
PTU	9.98	564676	----
Aniline	11.40	228556	----
Tyr	11.98	1340244	450.25
Val	12.70	2790862	960.13
Met	13.14	753814	323.62
Ile	14.43	1360616	521.45
Leu	14.60	3705736	1624.57
Phe	15.43	3253882	117.95
Lys	16.41	3545286	789.21
IPTU	17.17	340468	----
DIPTU	19.54	896444	----

Figure 11.9.12 Raw data from PTC-AAA. A typical PTC-AAA chromatogram and raw data reduction of peak area to picomoles of amino acid is shown for one analysis of a 36-kDa protein, human recombinant cellular retinaldehyde-binding protein (CRALBP; 1.9 µg hydrolyzed, 50% or 950 ng analyzed). Amino acids are identified using the three-letter code (see Table A.1A.1).

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Results from PROPSEARCH (http://www.embl-heidelberg.de/aaa.html)

Rank	ID	DIST	LEN2	POS1	POS2	pI	DE
1	cral_bovin	0.68	316	1	316	4.84	Cellular Retinaldehyde-Binding
2	cral_human	0.71	316	1	316	4.82	Cellular Retinaldehyde-Binding
3	cral_human	0.71	318	1	316	4.82	Cellular Retinaldehyde-Binding
4	y701_haein	1.79	339	1	339	5.87	Hypothetical protein HI0701
5	>p1;c64012	1.79	339	1	339	5.87	Hypothetical protein HI0701
6	spsd_bacsu	1.80	289	1	289	6.42	Spore Coat Polysaccharide
7	>p1;s39721	1.82	289	1	289	6.42	Hypothetical Protein-Bacillus
8	>p1;s43135	1.89	423	1	423	6.07	Serine-tRNA Ligase-Coxiella
9	sys_coxbu	1.89	423	1	423	6.07	Seryl-tRNA Synthetase
10	gba1_soybean	1.93	385	1	385	5.41	Gaunine Nucleotide-Binding

Results from ExPASy (http://expasy.hcuge.ch/ch2d/aacompi.html)

Rank	Score	Protein	pI	Mw	Description
1	6	CRAL_HUMAN	4.98	36343	Cellular Retinaldehyde-Binding
2	31	RO52_HUMAN	5.98	54169	52 kd RO Protein (Sjogren synd)
3	32	GRK6_HUMAN	8.33	65968	G Protein-Coupled Receptor Kinase
4	32	RYNR_HUMAN	5.19	64429	Ryanodine Receptor, Skeletal
5	36	CP27_HUMAN	8.43	56908	Sterol 26-Hydroxylase
6	37	SYD_HUMAN	6.20	57192	Aspartyl-tRNA Synthetase
7	38	VAV_HUMAN	6.20	98354	Vav Oncogene
8	38	STA5_HUMAN	5.98	90646	Signal Transducer
9	38	RFP_HUMAN	5.83	58489	Transforming Protein RFP
10	38	RTC1_HUMAN	6.34	23294	Ras-Like Protein TC21

Figure 11.9.13 Identification of CRALBP from PTC-AAA. The raw PTC-AAA data in Figure 11.9.12 from analysis of ~27 pmol recombinant human CRALBP was converted to mole % and entered into the PROPSEARCH and ExPASy database query programs for identification of the protein. The following mole % data were entered: Arg 7.07, Met 2.05, Glx 17.32, Ile 3.31, Ser 4.62, Leu 10.31, His 1.72, Phe 7.44, Gly 7.46, Lys 5.01, Thr 4.84, Asx 7.37, Ala 8.22, Pro 4.32, Tyr 2.86, Val 6.09, sum 100.01. The top ten scores from both programs are displayed. No species designation was used in the PROPSEARCH query; however, human was the designated species in the ExPASy search. The AAA data exhibit 6.9% compositional error. Abbreviations and scoring parameters are as in Figure 11.9.9.

and adjust response factors in the integrator. Data reduction of experimental samples using the computer-based spreadsheets and methods of calculation presented in this unit requires up to 1 hr for 5 to 10 analyses. Protein identification using the Internet and PROPSEARCH and ExPASy search programs typically requires 15 to 30 min per search. Allow another 15 to 30 min per day for general instrument maintenance.

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Reviews PTC-AAA methods and applications, including information about other precolumn derivatization methods and analysis of physiological fluid samples.

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N-Terminal Sequence Analysis of Proteins and Peptides

Amino-terminal (N-terminal) sequence analysis is used to identify the order of amino acids of proteins or peptides, starting at their N-terminal end. The complexity of the chemical reactions involved (see Fig. 11.10.1) has resulted in development of sophisticated instruments that process the protein or peptide in an automated manner. In order to undergo this cyclic process, an unmodified α-amino group is required at the N-terminal end of the molecule. After modification with phenylisothiocyanate (PITC), the derivatized terminal amino acid is removed by acid cleavage as its phenylthiohydantoin (PTH) derivative and a new α-amino group on the next amino acid is now available to react with

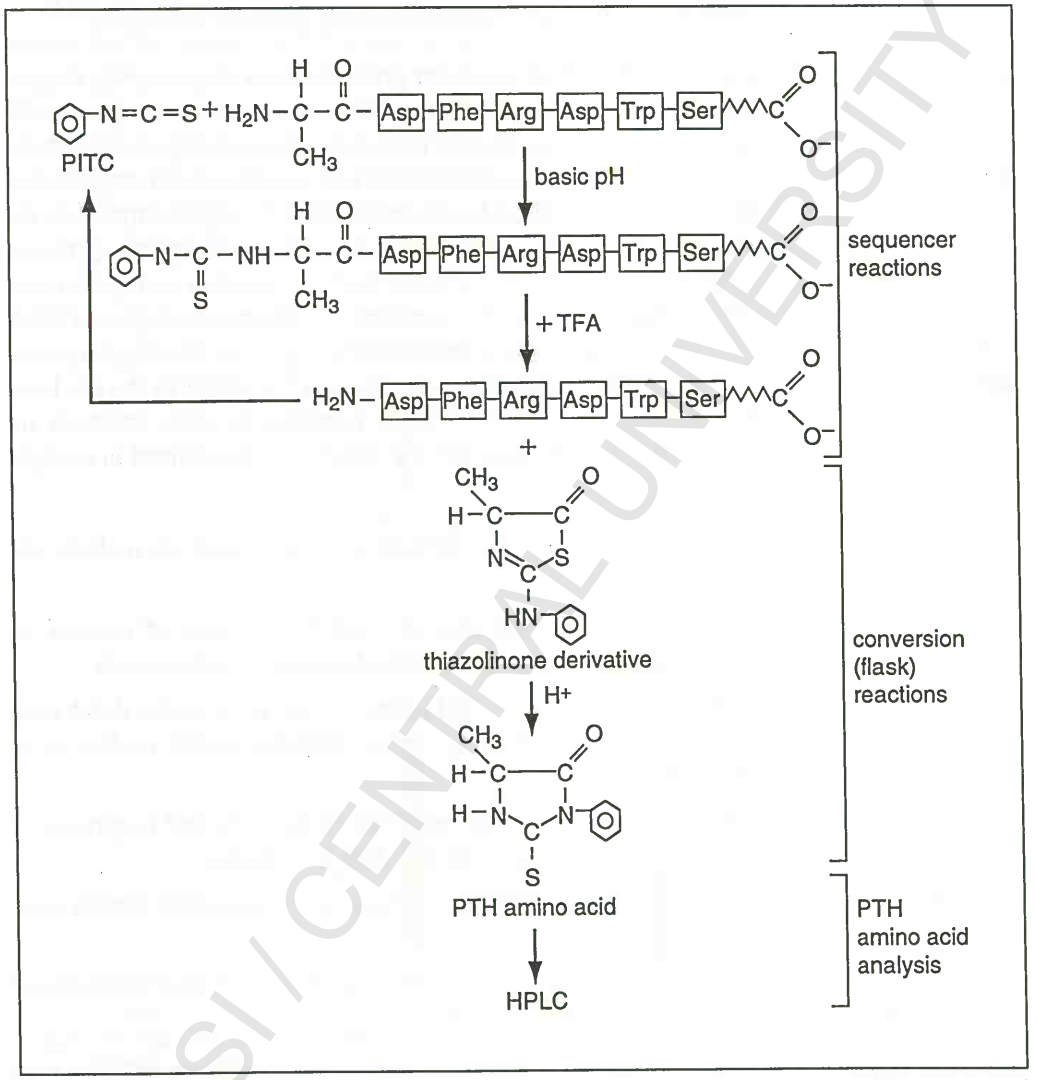

Figure 11.10.1 Edman chemistry and automated N-terminal sequence analysis. Modification of the free N-terminus of a protein or peptide sample with phenylisothiocyanate (PITC) at high pH, followed by acid cleavage of the modified terminal residue, results in release of a peptide one residue shorter in length and with a free N-terminus. This process represents a single sequencer cycle; subsequent residues can be removed by repeating this series of reactions (sequencer reactions). The cleaved 2-anilino-5-thiazolinone derivative (ATZ amino acid) is extracted from the sample support and transferred to a "flask" for conversion to the phenylthiohydantoin (PTH) amino acid (conversion reactions). The sample is then injected onto an HPLC column connected in line to the sequencer for separation analysis (PTH amino acid analysis).

Contributed by David F. Reim and David W. Speicher
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PITC. The series of sequencer reactions shown in Figure 11.10.1 represents a sequencing cycle that results in identification of the N-terminal amino acid present on the peptide or protein at the beginning of that cycle. If each step were 100% efficient, it would be possible to sequence an entire protein in a single sequencer run. In practice, multiple factors limit the amount of sequence information that can be obtained, and only rarely is it feasible to obtain >50 residues from a single sequencer run even when the amount of available protein is not limiting. With current technology, it is fairly routine to obtain at least 20 to 40 residues of sequence from the N-terminus of proteins and large peptides in the low picomole range (<50 pmol). However, a complication when working with an intact protein is that between 50% and 80% of all proteins are estimated to have a modified (blocked) N-terminal amino group and hence cannot be sequenced directly (Brown and Roberts, 1976). Several methods are available to deblock modified N-termini (see *UNIT 11.7*), although these methods usually require relatively large amounts of protein and may not always be successful, especially if the nature of the blocking group is not known.

Because most proteins have blocked N-termini, the preferred first step in analyzing an unknown protein is to proteolytically fragment the protein and obtain internal peptide sequences (see Strategic Planning). This multistep process involves separation of the protein of interest on either a one-dimensional (*UNIT 10.1*) or two-dimensional (*UNIT 10.4*) SDS polyacrylamide gel, possibly followed by electrotransfer to a polyvinylidene difluoride (PVDF) membrane (*UNIT 10.7*). This is followed by cleavage either in the gel (*UNIT 11.3*) or on the PVDF membrane with a specific protease such as trypsin or endoproteinase Lys-C (*UNIT 11.2*). Separation of the peptides is then accomplished by reversed-phase HPLC and the peptides are analyzed by MALDI mass spectrometry (*UNIT 11.6*). Finally, sequence analysis of one or more of the resulting peptides is carried out according to the methods in this unit (see Basic Protocols 1 and 3). Most peptides produced by these methods are <35 residues in length and their complete sequence can usually be determined in a single sequence run.

Currently, the most common applications for protein sequence analysis include the following.

1. Peptide sequences may be obtained and used to identify a protein of interest by searching them against protein and translated DNA databases (*UNITS 2.1 & 2.5*).
2. In the increasingly rare cases where the target protein is not in available databases, the peptide sequence may be used either to design oligonucleotide probes or to confirm putative cDNA clones.
3. The sequences may be used to characterize recombinant proteins and fragments of natural and recombinant proteins used in structure-function studies.
4. The sequences may be used to characterize recombinant proteins used as pharmaceutical reagents.
5. The sequences may be used to determine the sites and nature of post-translational modifications implicated in biological function.

This unit describes the sequence analysis of protein or peptide samples in solution (see Basic Protocol 1) or bound to PVDF membranes (see Basic Protocol 2) using a Perkin-Elmer Procise Sequencer. Sequence analysis of protein or peptide samples in solution (see Basic Protocol 3) or bound to PVDF membranes (see Basic Protocol 4) using a Hewlett-Packard Model G1005A sequencer is also described. (See *SUPPLIERS APPENDIX* for contact information regarding the manufacturers of these instruments.) Methods are provided for optimizing separation of PTH amino acid derivatives on Perkin-Elmer instruments (see Support Protocol 1) and for increasing the proportion of sample injected onto the PTH analyzer on older Perkin-Elmer instruments by installing a modified sample

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loop (see Support Protocol 2). The amount of data obtained from a single sequencer run is substantial, and careful interpretation of this data by an experienced scientist familiar with the current operation performance of the instrument used for this analysis is critically important. A discussion of data interpretation is therefore provided (see Support Protocol 3). Finally, discussion of optimization of sequencer performance as well as possible solutions to frequently encountered problems is included (see Support Protocol 4).

STRATEGIC PLANNING

Instrumentation

N-terminal protein/peptide sequencing instruments have improved dramatically over the past 30 years with the net effect that sequencer sensitivity has increased from sample amounts in the micromole level to the low picomole level. Modern sequencing instruments can be categorized into two groups based on the types of sequencer support that are used (Fig. 11.10.2). The most common sequencer support is a glass fiber filter (GFF). Most instruments in this group have been manufactured by Applied Biosystems (now owned by Perkin-Elmer), including the prototype Model 470A gas-phase sequencer initially produced in the early 1980s (Hewick et al., 1981). The latest models in this group are the Procise series Models 491, 492, and 494, which have one, two, or four sample cartridges, respectively. The availability of multiple sample cartridges allows automated

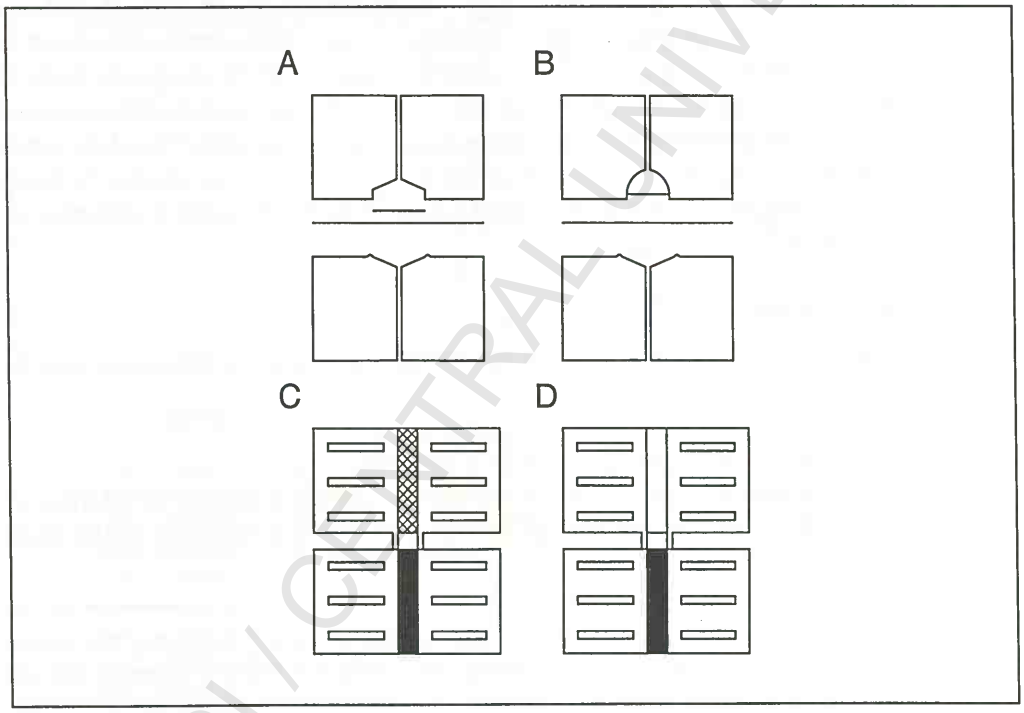

Figure 11.10.2 Sequencer sample cartridges. **(A)** Glass fiber filter (GFF) sample cartridge for a Perkin-Elmer sequencer, used to analyze samples in solution. The sample is loaded onto a Polybrene-treated GFF, inserted into the top reaction cartridge block, and the two halves of the cartridge are sealed with a Zitex (Teflon) cartridge seal. **(B)** Blott cartridge for a Perkin-Elmer sequencer, used to analyze proteins bound to PVDF membranes, which are inserted into the semicircular slot in the top of the Blott cartridge. **(C)** Biphasic sequencer reaction cartridge for a Hewlett-Packard G1005A sequencer, used to analyze samples in solution. The sample is loaded on the top half of the column (cross-hatched area) which contains a hydrophobic C18-type resin. The bottom half of the unit contains a hydrophilic resin (solid area). **(D)** A Hewlett-Packard sample cartridge for analyzing samples bound to PVDF membranes. The PVDF strips are inserted into an empty upper column (note absence of cross-hatched area). The bottom half of this unit is identical to the bottom half of the unit in panel C (solid area represents hydrophilic resin).

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tandem analysis of multiple sequence samples, which substantially increases instrument throughput and flexibility. The second type of sequencer is the Model G1005A (Hewlett-Packard) sequencer, which utilizes a biphasic sequencer sample support consisting of an upper column unit packed with a hydrophobic bonded phase on silica beads, and a lower hydrophilic column unit (Miller et al., 1990). This unique sample support is especially well suited to loading large-volume samples and/or samples contaminated with buffers or reagents that are incompatible with loading directly to a glass fiber filter support. This was the first commercial instrument to introduce a highly reproducible PTH analysis method using an ion-pairing reagent. Both the Perkin-Elmer and Hewlett-Packard have modified the design of the liquid sample cartridges (Fig. 11.10.2) to accommodate proteins bound to PVDF membranes (Sheer et al., 1991; Reim and Speicher, 1994). Although there are substantial differences between the two instruments in hardware design and the controlling software, differences in the overall sequencing sensitivity and sample throughput are relatively minor.

Both sequencer systems include integration and coordination of multiple components required for sequence analysis: i.e., a computer, the sequencer module, and a dedicated in-line HPLC for analysis of the PTH amino acid derivatives obtained from each sequencer cycle (PTH analyzer). The computer controls the overall operation of all components and handles data storage and analysis from the PTH amino acid analyzer. Figure 11.10.3, which is a schematic of the reagents, solvents, and flow paths for a Perkin-Elmer Procise Model 494 sequencer (with four sample cartridges) highlights the complexity of the instrument and the three major stages of sequence analysis: the Edman chemistry conducted on the sample support (sequencer reactions); sample conversion to a stable PTH derivative (flask reactions); and HPLC analysis (PTH analyzer). Each of these three stages takes a similar amount of time (~30 to 40 min), and to minimize total sample-analysis times, each of these three stages is performed in parallel. Therefore, while the first residue is being separated on the PTH analyzer, the second residue is being converted in the flask, and derivatization and cleavage of the third residue is occurring on the sample column.

Sample Preparation

To ensure the success of a sequencing experiment, the following considerations must be taken into account.

Should internal peptides or intact protein be sequenced?

When amino acid sequence information is needed to identify a protein of interest, an important decision is whether one should attempt an N-terminal sequence of the intact

Figure 11.10.3 *(at right)* Perkin-Elmer Procise 494 reagent schematic, illustrating the current complexity of instrumentation used for automated sequence analysis. Bottles for chemistry involved in sequencer reactions include: base (R2) and PITC (R1); trifluoroacetic acid (R3) for cleavage, which can be delivered in either the gas phase or as a small pulse of liquid reagent; solvents for removing reaction byproducts (S1 and S2) and extraction of the cleaved amino acid derivative to the conversion flask (S3); and additional reagent positions for addition of optional chemistries or solvents (X1 and X3). The conversion reactions section includes: aqueous acid (R4) for conversion of the amino acid derivative to PTH amino acids; acetonitrile/water (S4) to redissolve the PTH amino acids for HPLC analysis; PTH standard (R5) used for calibration; and additional positions for user-designated chemistries or solvents (X2 and X3). Delivery of appropriate chemicals or solvents to the sample cartridges or conversion flask are regulated by the related reagent or solvent valve blocks. The HPLC injector transfers the PTH amino acids from the conversion flask to an HPLC column. The HPLC pumps and detector are controlled through the sequencer computer during the sequencing process in the automated mode. Adapted with permission from Perkin-Elmer Procise User's Manual.

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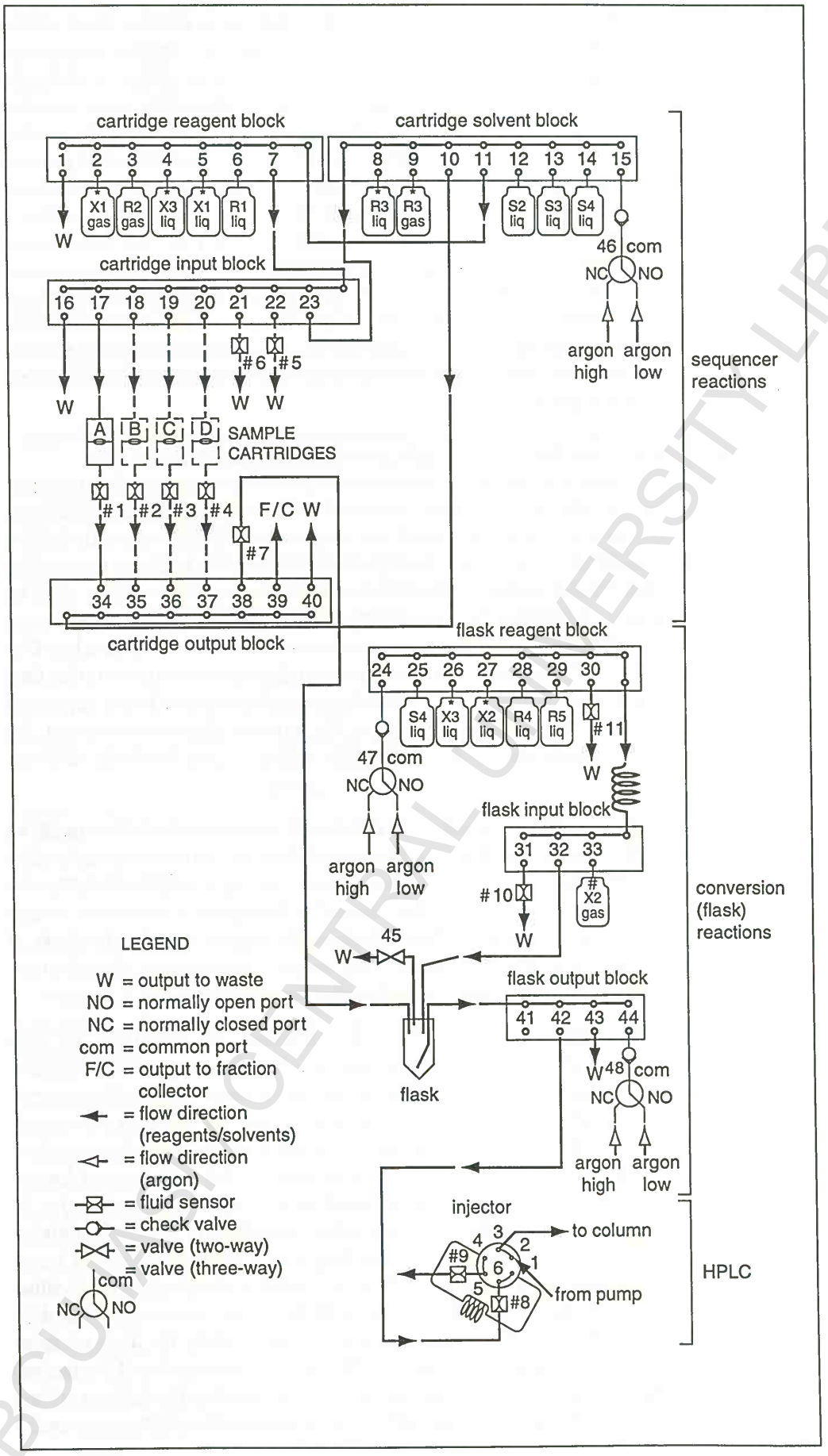

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protein or cleave the protein into peptides and determine internal sequences. Until several years ago, the large difference in the amount of sample needed for internal sequencing (>100 pmol) as compared to N-terminal sequencing (<10 pmol) tended to sway the decision for most sequencing laboratories toward initially attempting an N-terminal sequence, despite the high probability that the N-terminus would be naturally blocked and no sequence would be obtained. As noted above, between 50% and 80% of all proteins are naturally blocked and cannot be sequenced directly. However, improvements in producing and isolating proteolytic fragments in the low picomole range (see *UNITS 11.2, 11.3 & 11.6*) have reduced the amount of protein needed for obtaining internal sequences to the 10- to 20-pmol range for many sequencing laboratories. As a result, if the amount of protein that can be isolated is very limited, it is now usually advisable to initially attempt internal sequencing. Because similar amounts of protein are now required for either sequencing strategy, attempting internal sequencing provides a much higher probability of successfully obtaining the needed sequence information with the minimum investment of sample, effort, and expense.

Matching biological function with the right protein (the right band on a gel)

The most challenging protein isolation problems are those that arise where only a very limited amount of a protein can be isolated from a natural source. In the most typical case, a biological event or process is being studied and the goal is to identify a specific protein or proteins associated with a particular biological observation. In addition to isolating sufficient quantities of what is often a relatively low-abundance protein within a cell line or tissue, it is critical to ensure that the correct protein is isolated and sequenced. Although this latter point may seem so obvious that it does not deserve mention, a substantial portion of the samples submitted to sequencing laboratories actually are contaminants rather than the desired protein. Three helpful steps to ensure that the right protein is being sequenced are: (1) make an order of magnitude estimate of the amount of protein expected, (2) consider likely contaminants, and (3) run appropriate controls to test for likely contaminants.

Order of magnitude estimate: An order of magnitude estimate should be made to determine how much protein can feasibly be isolated from a given amount of source material. Even if little is known about the target protein, making a rough estimate about the protein's likely abundance may avoid effort wasted in attempting to isolate the protein from a source that can not possibly contain enough of the desired protein. Similarly, if the protein yields during purification dramatically exceed expectations, the observed protein band is probably a contaminant rather than the target protein.

As noted above, for those laboratories that have optimized the sensitivity of their procedures, the minimum amount of protein required for obtaining some sequence information from internal peptide sequencing is ~10 to 20 pmol. Although not every sequencing laboratory can analyze samples at this level, a 20-pmol minimum value will be assumed in this unit; the minimum value should be adjusted accordingly depending upon the anticipated sequencing sensitivity of the laboratory that will be used for the sequence analysis. There are 6×10^{23} molecules/mol or 6×10^{11} molecules/pmol $\times$ 20 pmol desired = 1.2×10^{13} molecules of purified protein required. To obtain the number of molecules that must be present in the initial sample this number is divided by an estimated overall purification yield—e.g., 0.25 (25% yield) for a high-yield purification involving only a few efficient steps such as an antibody affinity column followed by SDS-PAGE and electroblotting (lower overall yields would be likely for more complex purifications). With a 25% overall purification yield, 4.8×10^{13} molecules of the desired protein must be in the starting tissue. If the protein is to be purified from tissue culture cells, it is simple to estimate the number of cells required if some idea of the copy number

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Table 11.10.1 Estimating Cell Amounts Needed To Obtain Sequence Information[a]

Copies/ cell	Cells needed	Comments
10^6	5×10^7	Only the most abundant proteins including major cytoskeletal and structural proteins
10^5	5×10^8	Abundant proteins usually easy to detect on 2-dimensional gels of whole-cell homogenates
10^4	5×10^9	Generally below the detection limit on 2-dimensional gels of whole-cell homogenates
10^3-10^2	Not practical	Examples include many enzymes and regulatory proteins

[a]Based on a 25% overall purification yield and analysis by a sequencing laboratory capable of obtaining internal sequence information on 20 pmol of a target protein.

Table 11.10.2 Probable Protein Contaminants When Purifying Low-Abundance Proteins

Mol. wt. (kDa)[a]	Probable contaminants
200	Myosin
68	Serum albumin from source tissue or serum in tissue culture media
50-60	Intermediate filament proteins from source cells or human skin tissue keratins from airborne or direct-contact contamination
55	Antibody heavy chain[b]
43	Actin (usually the most abundant cell protein)
25-28	Antibody light chain[b]
Varied	Any added protein including enzymes such as DNase, RNase, soybean trypsin inhibitor, pancreatic trypsin inhibitor, or other protein-type protease inhibitors

[a]Approximate apparent size on an SDS gel run using reducing conditions.

[b]Definite major contaminant in immunoprecipitates and a likely contaminant when using an immunoaffinity column (column bleed).

per cell can be made (Table 11.10.1). These estimates illustrate that in most cases it is only feasible to use tissue culture cells as source material for major cellular proteins (>100,000 copies/cell). Purification of low-abundance proteins (100 to 1000 copies/cell) usually requires a substantial amount of a solid tissue; more purification steps are also often needed.

Contaminants and controls: The four most likely groups of protein contaminants are: (1) major cell proteins; (2) proteins used as reagents in the purification, such as antibodies; (3) proteins from culture media or serum proteins; and (4) keratins from skin. Residual contamination with a very small quantity of a major cell protein such as myosin or actin in a purified fraction can often exceed the amount of a lower-abundance target protein that has been recovered in high yield. A number of commonly observed protein contaminants are listed in Table 11.10.2. The best guard against focusing on a contaminant protein during isolation of a low-abundance protein is to analyze a parallel control sample that has been exposed to the purification conditions used, but which lacks the target protein. For example, if a cell extract is purified on a monoclonal antibody column, two useful controls can be run in parallel with the protein eluted from the specific antibody column. First, preelute the specific antibody column prior to the preparative purification with the same elution conditions as used to elute the target protein. Second, one can pass the cell

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extract through an unrelated monoclonal antibody column prior to the specific column and elute the nonspecific column using the same elution conditions. The controls and purified protein can then be analyzed in parallel on an SDS gel.

Avoiding chemical modification of the protein

It is especially critical to avoid the use of any reagent during the purification that might react with amino groups if the N-terminal amino group is not naturally blocked and sequencing of the intact protein is planned (e.g, to map a protease cleavage site or if the N-terminus is known to be unblocked). However, even if the planned strategy is to immediately pursue internal peptide sequences, it is very advisable to avoid any chemical modification of reactive groups on the protein. Modification of side-chain amino groups on lysines will interfere with trypsin or endoproteinase Lys-C digestion, and modification of *any* reactive groups on the protein will introduce heterogeneity that may reduce yields during protein purification steps. This microheterogeneity will reduce the yields of specific peptides and increase the complexity of HPLC peptide maps, as the different chemical forms of each peptide will probably be separated from each other at the reversed-phase HPLC step.

The reagent most frequently used in protein purifications that leads to protein modification is urea (also see *APPENDIX 3A*). Although urea itself is uncharged and does not directly react with proteins, it decomposes rapidly to form cyanate, which reacts with amino groups. If possible, urea should be eliminated from the purification procedure. If its use cannot be eliminated, the following precautions should be taken: (1) purchase an "ultrapure" grade of urea (the dry powder is stable at room temperature; solutions are *not* stable); (2) prepare urea-containing solutions using ultrapure urea immediately before use and keep the temperature as low as possible when using the solution; (3) include a scavenger in the urea solution that will react with cyanate as it forms, e.g., 200 mM Tris·Cl or 50 mM glycine; (4) keep the pH at 7.0 or lower (if feasible), as amino groups are ~10-fold more reactive at pH 8.0 than at pH 7.0 and cyanate is less stable at acidic pHs. For example, it should be safe to expose a protein to a 7 M urea solution containing 50 mM glycine at pH 7.0 for at least 24 hr at 4°C or for several hours at room temperature. Note that an appropriate buffer such as sodium phosphate should be included in this solution, as neither glycine nor Tris·Cl have much buffering capacity at pH 7.

Avoiding adsorptive losses

It is well known that small amounts of proteins and peptides adsorb to glass and plastic surfaces and various strategies are commonly employed to minimize such losses. Adsorptive losses are especially problematic in late stages of purifications where low pmol amounts (low μg amounts) of proteins are being isolated for sequence analysis. Addition of a blocking protein such as 0.1% BSA to solutions is not feasible, as minor contaminants and cross-linked forms of the blocking protein are almost certain to interfere with sequence analysis of the target protein even if the target protein has a very different molecular weight.

The severity of protein adsorptive losses is often underestimated. As shown in Table 11.10.3, appreciable protein adsorption to both glass and plastic tubes occurs within seconds. The adsorbed protein is tightly bound to the container surface and can only be removed with very harsh conditions, e.g., 1% SDS. Because the values in Table 11.10.3 represent brief exposure of a dilute protein to a single tube, the actual adsorptive losses that can occur during late stages of a purification would be expected to be far higher than those shown here. The best method of minimizing such losses is to add a detergent, preferably SDS, to the protein sample in late purification steps; this is likely to be especially important for any step where the total protein concentration of the sample is

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Table 11.10.3 Adsorptive Losses of Proteins from Dilute Solutions[a]

Protein	Glass test tube		Siliconized glass test tube		Polypropylene microcentrifuge tube	
	µg	pmol	µg	pmol	µg	pmol
Phosphorylase B	0.4	4.3	0.7	7.5	0.6	6.4
BSA	1.2	18	2.0	30	0.2	3.0
Ovalbumin	0.9	21	1.3	30	0.3	6.9
Myoglobin	1.9	112	2.5	147	1.0	58

[a]Amount of protein lost by adsorption to the indicated container surface within 15 sec of addition of a 300-µl aliquot of dilute protein solutions in phosphate buffer, pH 7. Samples were pipetted directly into the bottoms of the tubes to minimize exposure to the container surfaces. Losses were determined by quantitative amino acid analysis of the protein left in solution. Protein adsorbed to the container surface could be extracted using 1% SDS. Adsorptive losses were higher than the values shown here when the solution was vortexed or when protein solutions were applied to the top of the tube and allowed to run down the side.

<0.1 µg/µl. As discussed below, the final purification step should be an SDS gel, therefore the addition of SDS to the sample during late steps of most purifications should be feasible. Of course, biological activity will be irreversibly lost in most cases upon addition of SDS, and if maintaining biological activity is critical for monitoring purification steps, an alternative blocking reagent such as a nonionic detergent (e.g., Triton X-100 or Tween 20) should be considered.

Final purification step

SDS-PAGE gels are the preferred final purification step for most protein sequencing applications. SDS gels are ideally suited for handling low microgram to submicrogram amounts (low picomole levels) of protein. They have high resolving power, and most buffer contaminants that would interfere with direct sequencing or with a protease digestion are separated from the target protein in the gel. Protein losses resulting from adsorption are negligible in comparison to those incurred with virtually any other method, and SDS gels are compatible with addition of SDS or another detergent to samples in preceding purification steps to minimize adsorptive losses (see above). After SDS-PAGE, the protein can be electroblotted to a high-retention PVDF membrane (*UNIT 10.7*) for direct N-terminal sequencing (see Basic Protocols 2 and 4) or else the protein can be digested on the membrane with trypsin, endoproteinase Lys-C, or another protease to obtain internal peptides (*UNIT 11.2*) for sequence analysis. Alternatively, the protein can be digested with a protease directly in the gel (*UNIT 11.3*). Another advantage of isolating proteins in this manner is that the protein in the gel or on the PVDF membrane is in a denatured form that facilitates proteolytic digestion.

Because SDS gels are usually used as the final purification step for sequence analysis, the protein of interest does not need to be purified to homogeneity by other methods. In some cases, quite crude extracts have been applied to a one-dimensional gel and the protein of interest identified. The limitations to this approach are that: (1) the migration position of the protein of interest on the gel must be known, (2) the target protein must be resolved from other proteins in the sample by the gel system used, and (3) the target protein must be present in the sample in a sufficiently high proportion so that contaminating proteins do not cause a gel-overload condition when sufficient target protein for sequence analysis is applied to the gel. Complex samples that may contain multiple proteins at the target-protein migration position on a one-dimensional gel can usually be adequately separated by two-dimensional gel electrophoresis (*UNIT 10.4*), and the target protein from one or several replicate two-dimensional gels (or electroblots from the

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two-dimensional gels) can be combined and digested with trypsin. Frequently, high-abundance proteins can be isolated for internal sequencing by loading a whole-cell homogenate directly to a series of replicate two-dimensional gels. However, despite the very high resolving power of two-dimensional gels, a single spot on a two-dimensional gel may contain multiple proteins, especially when very complex samples such as whole-cell extracts are used.

A useful guideline is to purify proteins larger than ~6 kDa using SDS-PAGE as the last purification step; peptides smaller than 6 kDa from either in situ protease digests or from other sources should be purified using reversed-phase HPLC (*UNIT 11.6*). If <100 pmol of peptide is being isolated, use of a 1.0- or 2.1-mm-diameter C18 column with acetonitrile and 0.1% TFA as the reversed-phase solvents is recommended.

Amount of protein used for sequence analysis and observed sequencer yields

Occasionally, enthusiastic sequencer manufacturers as well as research scientists claim that 1 pmol or less of a protein can be sequenced. A critical point that is often not emphasized is the cited sensitivity usually refers to the *observed* sequence yield in early sequencer cycles, not the *total* amount of protein or peptide actually used in the experiment. The average initial sequence yield for proteins or peptides (i.e., the signal observed in early sequencer cycles divided by the amount loaded into the sequencer multiplied by 100) averages ~50% (with the typical range 20% to 80%). Because adsorptive losses and artifactual modification of the N-terminus are likely to increase as the quantity of sample decreases, where 1 pmol of a protein or peptide is loaded into a sequencer, a realistic initial yield would be 0.1 to 0.5 pmol. Only a few laboratories that have carefully optimized all aspects of the sequence analysis procedure can obtain significant N-terminal sequence information at this level.

Obtaining internal sequences involves multiple steps that are individually reasonably efficient but that have the effect of further reducing the observed sequence yield relative to the starting protein. A reasonable guideline is that initial sequencing yields of internal peptides relative to the amount of a protein loaded onto a gel is ~15% (with a typical range of 5% to 30%). This means that 20 pmol of a target protein applied to a gel will yield internal sequences with initial yields between 1 and 6 pmol. Although these overall yields may appear to be quite poor, the yields at each step of the procedure must be quite good to achieve such overall yields. An example of yields at each step that might be obtained in a laboratory that has carefully optimized the procedure might be as follows:

- recovered in the major gel band relative to the amount applied to the top of the gel—90%
- electroblotting efficiency—70%
- trypsin digestion efficiency and recovery of cleaved peptides from either gel or PVDF membrane—70%
- recovery from the HPLC—90%
- recovery from the collection tube—80%
- initial yield in the sequencer—50%.

The cumulative yield in this example would be: $0.9 \times 0.7 \times 0.7 \times 0.9 \times 0.8 \times 0.5 = 0.16$, or 16%.

Estimating amount of protein used for sequence analysis

Estimating the amount of target protein that has been isolated and that will be used for either obtaining a N-terminal sequence or internal sequences contributes to proper interpretation of results. For example, if a protein band that has been electroblotted to a PVDF membrane does not produce a detectable sequence, knowing the amount loaded

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into the sequencer should indicate whether the protein was blocked or insufficient protein was present. Alternatively, a low-level sequence may arise from a minor contaminant rather than the target protein, which might be blocked. For example, assuming that a PVDF-bound band yielded a 2-pmol sequence, if ~4 pmol were present on the membrane, this sequence would clearly correspond to the major component in that band, but if 40 pmol were present it would be considered likely that the major component was blocked and that a minor contaminant in the gel band produced the minor sequence observed.

Ideally, accurate quantitation of a portion of the protein or peptide sample should be made prior to either N-terminal sequencing or protease digestion. Unfortunately, when only low picomole amounts of protein are available, a substantial portion of the total sample is required simply to obtain a reasonable estimate of protein concentration by amino acid analysis (AAA), which is the most reliable method of quantitating protein concentrations in solution, in gels, or on PVDF membranes (see *UNITS 3.2, 11.3 & 11.9* for AAA and related techniques). Therefore, if an unacceptable proportion of the sample would be consumed by quantitative AAA, the amount of protein on the gel or PVDF membrane can be estimated by comparing its staining intensity to the staining intensity of a series of standard protein lanes prepared using 2-fold dilutions—e.g., adjacent lanes containing 2 μg/band, 1 μg/band, 0.5 μg/band, 0.25 μg/band, and 0.125 μg/band. If the protein standards are run on the same gel as the experimental sample, one can readily estimate the amount of target protein available for sequencing to within a factor of ~2 to 4, with the important limitation that there is some variability in staining response for different proteins. However, Coomassie blue, the preferred stain for in-gel digestions (*UNIT 11.3*), and Amido black, the preferred stain for on-PVDF digestions (*UNIT 11.2*) show much less protein-specific variability in staining than silver staining.

MALDI mass spectrometry

If access to a mass spectrometer is available, it is highly advantageous to prescreen all peptides prior to sequence analysis using MALDI mass spectrometry (*UNITS 11.6 & 16.2*) to complement peptide sequence analysis. Mass analysis of ~10 to 15 peaks from HPLC separation of an in situ trypsin digest is recommended. The longest peptides (i.e., with the largest masses) should be selected for sequencing, as longer sequences provide more informative database-search results, especially when related proteins, but not the identical protein, are in the sequence database searched. Similarly, if the protein is not known, longer sequences provide a wider range of options for oligonucleotide probe design. After the sequence run is complete, comparison of the assigned sequence with the previously determined mass provides confirmation of the residue assignments and can extend interpretation of the sequencer data if most, but not all, of the residues in the peptide are clearly assigned. For example, reconciliation of the mass with the sequence can confirm one or two tentative assignments, suggest possibilities for an unassigned residue, or indicate the presence of a post-translational modification. In theory, prescreening HPLC peptides by MALDI mass analysis can also potentially identify peptide mixtures. In practice, only some mixtures are detected by this method, because mass signals obtained by this method are not quantitative and some peptides can suppress the signals of other peptides.

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BASIC PROTOCOL 1

SEQUENCING LIQUID SAMPLES ON GLASS FIBER FILTERS

This protocol describes application of a highly purified protein or peptide in solution to a Perkin-Elmer Procise model 494 sequencer. Sample preparation and loading procedures would be similar for other sequencers that use glass fiber filter (GFF) supports. The sample should ideally be in a small volume of high-purity water or a volatile buffer/solvent.

Materials

Liquid peptide or protein sample(s) to be sequenced
Volatile solvents for reversed-phase chromatography: e.g., 0.1% trifluoroacetic acid (TFA) and acetonitrile (*UNIT 11.6*)
Methanol
Argon or nitrogen gas source
Polybrene solution (Biobrene Plus from ABI Biotechnology; store in original container up to 3 months at 4°C; discard if contamination is suspected; alternatively store 30- to 100-µl aliquots in clean microcentrifuge tubes up to 1 year at –20°C)
Sequencer solvent/reagent kit (ABI Biotechnology) including:
- R1 (phenylisothiocyanate)
- R2B (*n*-methylpiperidine)
- R3 (trifluoroacetic acid)
- R4A (25% trifluoroacetic acid)
- R5 (PTH sequencing standard)
- S2B (ethyl acetate)
- S3 (*n*-butyl chloride)
- S4B (20% acetonitrile)

Premix PTH analyzer kit (ABI Biotechnology) including:
- HPLC Solvent A3 (3.5% tetrahydrofuran)
- HPLC Solvent B2 (isopropanol/acetonitrile)
- Premix Buffer Concentrate
- DMPTU

1.0- or 2.1-mm-i.d. reversed-phase column (*UNIT 11.6*)
TFA-treated glass fiber filters (GFF; ABI Biotechnology)
Cartridge seals (ABI Biotechnology)
Perkin-Elmer Procise sequencing system Model 494 (ABI Biotechnology) including:
- Sequencer module with glass sample cartridge blocks
- On-line PTH analyzer (dedicated HPLC and detector)
- Computer controller

Powder-free gloves
Stainless steel or Teflon-coated forceps

Additional reagents and equipment for reversed-phase purification of peptides (*UNIT 11.6*), concentration of proteins and microdialysis (*UNIT 4.4*), quantitation of proteins/peptides by amino acid analysis (*UNITS 3.2 & 11.9*), and spectrophotometric quantitation of protein (*UNIT 3.4*)

NOTE: Prepare solutions with Milli-Q water or equivalent taken directly from the purification unit. Water stored for any length of time in glass or plastic containers supports microbial and algal growth, which results in high amino acid background in early sequencer cycles.

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1. Prepare peptide or protein samples in a small volume (preferably <50 µl) of high-purity water or volatile buffer/solvent.

   Small amounts (<10 µmol) of nonvolatile, non-amine reactive salts such as NaCl can usually be tolerated, but at higher levels, salts may interfere with binding of the protein or peptide to the GFF and can interfere with retention times of His and Arg in early cycles. Nonvolatile buffers can interfere with sequencer performance by altering the actual pHs achieved during the coupling (basic pH) and cleavage steps (acid pH). Any nonvolatile components or contaminants such as Tris buffer that react with amine-specific reagents are especially problematic.

   Larger volumes can be reduced in a Speedvac evaporator, but this will concentrate nonvolatile impurities and it is likely to increase adsorptive losses on the tube holding the sample.

Purify and/or concentrate sample

2a. *If analyzing a peptide sample:* Purify the peptide on a 1- or 2.1-mm-i.d. reversed-phase column using volatile solvents such as 0.1% TFA and acetonitrile (*UNIT 11.6*).

2b. *If analyzing a protein sample:* Concentrate the sample to ~100 µl, then desalt the sample by exhaustive microdialysis (*UNIT 3.4*) against water taken directly from a Milli-Q purification unit or equivalent.

Estimate amount of protein or peptide sample

3a. *If >100 pmol of sample is available:* Use 20% of the protein or peptide sample for quantitative amino acid analysis using a high-sensitivity method such as precolumn phenylthiocarbamyl (PTC) derivatization (*UNIT 11.9*).

   This will accurately define the amount to be loaded and detect potential contaminants in the sample that can react with amine reactive reagents.

3b. *If <100 pmol of sample is available:* Estimate the quantity of sample to be loaded by an indirect method such as absorbance at 280 nm (for proteins) or 215 nm (for peptides) as described in *UNIT 3.4*.

Precycle glass fiber filter (GFF)

4. Thoroughly rinse the glass sample cartridge blocks with methanol and dry with a stream of argon or nitrogen while wearing powder-free gloves that have been prerinsed with Milli-Q water.

   Refer to manufacturer's instructions for the Procise system for this and all subsequent steps.

   Do not touch with bare hands any surfaces that are part of the sequencer chemistry flow path or that will contact the samples. These include, e.g., the glass sample cartridge, the GFF, solvent pickup tubes, and bottle seals. Also avoid exposing these surfaces to airborne contamination.

5. Place a TFA-treated GFF on the top half of the cartridge block with a pair of stainless steel or Teflon-coated forceps. Saturate filter by applying 15 µl (for 9-mm filters) or 30 µl (for 12-mm filters) of Polybrene solution.

6. Dry Polybrene solution completely using a gentle argon or nitrogen stream, or air dry GFF under a piece of aluminum foil to prevent dust from settling on it.

7. Assemble the two cartridge halves using a new cartridge seal and insert the assembled unit into the sequencer. Pressure test the cartridge to detect leaks.

8. Run at least two cycles using the appropriate filter-conditioning program.

   Samples can be loaded immediately after completing the conditioning cycles or the GFF can be stored in a gas-tight bottle up to 3 days. However, the increased handling involved

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in removing the filter from the sample cartridge and reinserting it after storage can result in an increased background in early sequencer cycles, especially for Ser and Gly.

The filter-conditioning program can also include a flask program that injects the PTH standard so that the quality of the PTH amino acids can be evaluated. Alternatively, a flask blank can be injected on the HPLC during filter-conditioning cycles.

After the conditioning cycles have been completed and prior to loading the sample, one complete sequencing cycle can be run to evaluate any background that is contributed by the Edman sequencing chemistry.

Apply sample and perform sequence analysis

9. Remove the sample cartridge from the sequencer (wearing powder-free gloves) and apply 15 to 30 µl (depending upon filter diameter) of the sample to the GFF in the upper half of the sample cartridge using a pipettor. Dry the filter using a gentle stream of argon or nitrogen, then repeat application and drying until the entire sample has been applied. Alternatively, air dry the sample between repetitive applications using an aluminum foil "tent" to prevent airborne contaminants from settling on the GFF.

   Do not overload the filter with more liquid than it can hold. Most sample volumes are larger than the filter capacity, hence the need for repetitive applications. The number of applications will depend on the total volume of the sample being loaded (volumes >100 µl are quite time-consuming to load and increase the risk of contamination).

   If HPLC-purified peptide samples containing <20 pmol are used for sequence analysis, addition of trifluoracetic acid (25% final concentration) to the sample may increase the amount of sample actually transferred into the sequencer (Erdjument-Bromage et al., 1993). Estimate the sample volume by comparing the liquid height to an identical tube calibrated by marking volumes bracketing the expected volume on the tube. Add a volume of neat TFA (reagent R3 from ABI Biotechnology) equal to one-third the sample volume (25% TFA final concentration). It is very important not to vortex the sample! Use a pipettor to mix the TFA/peptide solution completely. After loading the entire sample, wash the sides of the empty sample tube with 20 µl neat TFA and apply to the sample filter.

10. After the sample has been completely loaded and dried, place a new cartridge seal on the bottom glass block, reassemble the sample cartridge, and insert the assembly into the sequencer. Pressure test the cartridge assembly to detect leaks.

11. Check the levels of all needed items—e.g., sequencing reagents, PTH analyzer solvents, argon, and printer paper—to ensure that sufficient quantities are available to complete the projected number of sequence cycles. Program the computer appropriately using manufacturer's recommendations and carry out the sequence run.

    At least one PTH standard or a PTH analyzer blank run followed by a PTH standard should be injected immediately prior to each sequence run to verify retention times and to determine calibration factors for each amino acid. The number of Edman cycles that should be run depends upon the characteristics of the sample and the purpose of the analysis. For most applications, it is recommended that peptides be sequenced through their C-terminus plus two cycles (to ensure that the entire sequence has been obtained and to assess background levels of amino acids; see Support Protocol 3). If the peptide's mass has been determined prior to the sequence run, the number of cycles to run can be estimated by: cycles = (peptide mass/100) + 3 (rounded up to next integer). For example, if the observed mass is 1644.5 Da, the sequencer can be set to complete 20 sample cycles. When intact proteins or large peptides are being analyzed and a maximum amount of sequence data is desired, set a number of cycles greater than the number that will be completed in an overnight run; the next morning review the data and continue the sequence until the decreasing signal-to-noise level limits sequence assignments (see Support Protocol 3). If the protein sequence is known and the purpose is to verify an intact N-terminal sequence or to define a cleavage site, 5 to 6 cycles should be sufficient. If the protein is to be searched

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against sequence databases to identify the protein, analysis of at least 20 cycles is recommended.

Procise sequencers can be programmed to automatically analyze up to four samples in tandem. Therefore, care must be taken to ensure that sufficient reagents/solvents are available for the total cycles programmed and that the appropriate sequencing programs have been selected for the sample(s) to be analyzed.

SEQUENCING PVDF-BOUND SAMPLES USING A BLOTT CARTRIDGE

BASIC PROTOCOL 2

For most applications where N-terminal sequence analysis of intact proteins or large peptides (>6 kDa) is desired, the preferred final purification method is to use one-dimensional (*UNIT 10.1*) or two-dimensional (*UNIT 10.4*) gels followed by electroblotting to a high-retention PVDF membrane (*UNIT 10.7*) and staining with Amido black (*UNIT 10.8*). For more detailed discussion of these preliminary steps, see Strategic Planning. PVDF membranes have a high protein-binding capacity, bind large peptides and proteins with high affinity, and are inert to the harsh chemical environment in a protein sequencer. However, the wetability characteristics of these hydrophobic membranes are quite different from those of the hydrophilic glass filters (see Basic Protocol 1). These differences have necessitated development of specific sequencer programs and sample holders for PVDF membranes (see Fig. 11.10.2). The preferred method for loading proteins onto a PVDF membrane is by electroblotting from a gel. Dilute samples can be concentrated and/or separated from an incompatible buffer by direct adsorption to PVDF membranes. If detergents are not present in the protein solution, the protein can be adsorbed by immersing a small piece of PVDF membrane (which must be prewetted in 100% methanol and rinsed with water) into the sample solution and incubating it with agitation in a cold room for several hours to overnight. Alternatively, the sample can be loaded onto a PVDF disc in the bottom of a ProSorb device (ABI Biotechnology). Directly spotting a protein sample onto a prewetted PVDF membrane is not recommended as this is an unreliable loading method. The total liquid volume that can be applied to the PVDF membrane is quite small, and adsorption to the PVDF membrane competes with sample drying. After the liquid has dried, further adsorption does not occur. When the sample is rewet, either prior to loading in the sequencer or during sequence analysis, the dried protein that did not bind is usually washed from the membrane, which results in reduced sequencing yields.

Materials

PVDF-bound sample(s) to be sequenced
Methanol in wash bottle
Argon or nitrogen gas source

Glass gel plate
Stainless steel scalpel and stainless steel or Teflon-coated forceps
Bath sonicator
Blott cartridge for Perkin-Elmer Procise sequencer (ABI Biotechnology)

Additional reagents and equipment for sequencing on glass fiber filters (see Basic Protocol 1)

NOTE: Prepare solutions with Milli-Q water or equivalent taken directly from the purification unit. Water stored for any length of time in glass or plastic containers supports microbial and algal growth, which results in high amino acid background in early sequencer cycles.

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Prepare sample

1. Using a scalpel, excise the desired stained protein band from PVDF membrane, keeping the membrane on a clean dry surface such as a glass gel plate and handling the membrane with a forceps.

   The glass plate, scalpel, and forceps should be thoroughly cleaned before use with Milli-Q water and methanol. Wear powder-free gloves rinsed in Milli-Q water. Do not touch the membrane or surfaces that contact the membrane with bare hands.

   Dry PVDF membranes are electrostatic and can be difficult to handle. The electrostatic nature of the membranes also attracts airborne contamination that invariably contains amino acid and protein contaminants. Special caution must be exercised to minimize contamination of the sample when working with small amounts of protein (<20 pmol).

   Two to three replicate 3-mm-wide one-dimensional gel bands on PVDF (50 to 75 mm^2 in area) or an equivalent area of membrane containing replicate two-dimensional gel spots can typically be loaded into a Procise cartridge. The larger Blott cartridge on older Perkin-Elmer sequencers can accommodate about twice as much membrane surface area.

2. While holding the membrane(s) with stainless steel or Teflon-coated forceps, thoroughly rinse with a stream of methanol from a wash bottle. Immediately rinse the membranes with a stream of Milli-Q water delivered directly from the Milli-Q purification unit.

   PVDF membranes cannot be solvated directly with aqueous solutions and must initially be wetted in a strong organic solvent such as methanol. After the membranes are wetted with methanol in the above step, do not allow them to dry out until drying is indicated in step 3.

3. Place the membrane(s) in a clean beaker containing 5 ml Milli-Q water and sonicate 5 min in a bath sonicator. Remove the membrane(s) with forceps, rinse with methanol, and dry under a gentle stream of argon or nitrogen.

Load sample and perform sequence analysis

4. Insert the membrane(s) in the slot in the top half of the Blott cartridge.

   Refer to the manufacturer's instructions for this and subsequent steps.

   If needed, an additional piece of membrane can be placed protein-side-up within the recess on the top half of the cartridge.

5. Reassemble the Blott cartridge using a new cartridge seal and insert the assembly into the sequencer. Pressure test the cartridge assembly to detect leaks.

6. Check the levels of all needed items—e.g., sequencing reagents, HPLC solvents, argon, and printer paper to ensure that sufficient quantities are available to complete the projected number of sequence cycles. Program the computer appropriately using manufacturer's recommendations and carry out the sequence run.

   At least one PTH standard or a PTH analyzer blank run followed by a PTH standard should be injected immediately prior to each sequence run to verify retention times and to determine calibration factors for each amino acid. The number of Edman cycles that should be run depends upon the purpose of the analysis. When intact proteins are sequenced and a maximum amount of sequence data is desired, set a number of cycles greater than the number that will be completed in an overnight run; the next morning review the data and continue the sequence until the decreasing signal-to-noise level limits sequence assignments (see Support Protocol 3). If the protein sequence is known and the purpose is to verify an intact N-terminal sequence or to define a cleavage site, 5 to 6 cycles should be sufficient. If the protein is to be searched against sequence databases to identify the protein, analysis of at least 20 cycles is recommended.

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SEQUENCING LIQUID SAMPLES ON A BIPHASIC CARTRIDGE SEQUENCER

The unique sample cartridge in the Hewlett-Packard sequencer (Fig. 11.10.2) results in quite different sample loading constraints than those encountered with sequencers utilizing a glass fiber filter (see Basic Protocol 1). Since samples are loaded onto the hydrophobic half of the cartridge unit, sample loading constraints are similar to those involved in applying a sample to a C18 reversed-phase column (also see *UNIT 11.6*). Large volumes of aqueous samples can be readily loaded. Most buffers, as well as high levels of nonvolatile salts that would severely interfere with sequence analysis on a glass filter, are easily accommodated because any hydrophilic buffers including those containing Tris and glycine can be readily washed out of the column. Because the binding of proteins or peptides to the sample cartridge is via hydrophobic interactions, only strong organic solvents or detergents are likely to interfere with sample binding. Samples in high concentrations of organic solvents can usually be successfully loaded by simply diluting the sample with 2% TFA or Milli-Q water to reduce the solvent strength, unless the peptide does not remain soluble in the more hydrophilic solvent. Samples in detergents may bind to the column either directly or after the sample is diluted; however, efficient sample loading should not be assumed in this case and the unbound fraction should be analyzed by an appropriate method such as quantitative amino acid analysis (*UNIT 11.9*), if feasible, or using SDS gels to detect unbound proteins and HPLC to detect unbound peptides.

Materials

Methanol
Source of argon or nitrogen
Liquid peptide or protein sample(s) to be sequenced
Sequencer and PTH analyzer solvent/reagent kit (Hewlett-Packard) including:
- R1 (phenylisothiocyanate)
- R2 (diisopropylethylamine)
- R2A (octylamine)
- R3 (trifluoroacetic acid)
- R4 (25% trifluoroacetic acid)
- S2A (ethyl acetate)
- S2/3 (23% acetonitrile/77% toluene)
- S3 (15% acetonitrile/85% toluene)
- S3A (aqueous trifluoroacetic acid solution)
- S4 (10% acetonitrile)
- Std (PTH sequencing standard)
- L1 (acetonitrile)
- L2 (methanol)
- HPLC solvent A (aqueous triethylamine/acetonitrile)
- HPLC solvent B (aqueous triethylamine/propanol)
- HPLC solvent C (acetonitrile)

Sample cartridges for liquid samples (Hewlett-Packard)
Sample loading funnels (Hewlett-Packard)
Hewlett-Packard Model G1005A sequencing system including:
- Sequencer module
- On-line PTH analyzer (dedicated HPLC and detector)
- Computer controller
- Sample loading station

Powder-free gloves

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NOTE: Refer to the manufacturer's instructions for the Hewlett-Packard Model G1005A sequencing system at all steps.

NOTE: Prepare solutions with Milli-Q water or equivalent taken directly from the purification unit. Water stored for any length of time in glass or plastic containers supports microbial and algal growth, which results in high amino acid background in early sequencer cycles.

1. Precondition the sample cartridge in the sequencer using the version 2.0 column preparation program for one cycle.

   Sample cartridges can be preconditioned and stored in a dry, closed container ≤1 week. Airborne contamination and sample handling contamination are less of a concern with this sample unit than when working with a glass fiber filter (see Basic Protocol 1) because of its design (i.e., it does not expose a large sample surface area). Mark the cartridges with the date immediately after preconditioning using a permanent laboratory marker.

   Newer versions of the Hewlett-Packard column preparation program are ~40 min in length but do not exhibit apparent advantages over the shorter (~20-min) version 2.0 program. The shorter column preparation program is therefore recommended.

2. Prepare the sample loading funnel prior to inserting it in the sample loading station by rinsing extensively with ~30 ml Milli-Q water and then rinsing a large number of times with methanol (~30 ml total). Fill the funnel with one volume of sample loading solution and allow to completely drain through the funnel. Dry the funnel with a gentle stream of argon or nitrogen.

   Use powder-free gloves that have been rinsed with Milli-Q water when cleaning the loading funnel and applying samples to the sample cartridges. In all instances, avoid contacting the inside of the sample loading funnel and the ends of the sample cartridges with bare fingers.

3. Attach the top half of a conditioned sample cartridge to a washed sample loading funnel and insert into the sample loading station. Add 1 ml methanol to the loading funnel and apply nitrogen pressure to force the methanol through the sample column. Release the pressure when ~5 to 10 µl remains in the funnel. Add 1 ml of sample loading solution to the funnel, then force the liquid through the sample column until ~5 to 10 µl remains in the funnel.

4. Estimate the volume of the sample to be analyzed. Add an appropriate volume of sample loading solution to the funnel, calculated as follows: 1.0 ml – sample volume = sample loading solution volume.

   If HPLC-purified peptide samples containing <20 pmol are used for sequence analysis, addition of TFA (25% final concentration) to the sample may increase the amount of sample actually transferred into the sequencer (Erdjument-Bromage et al., 1993). Estimate the sample volume and add neat TFA (reagent R3 from ABI Biotechnology) equal to 25% TFA final concentration. It is very important not to vortex the sample! Use a pipettor to mix the TFA/peptide solution completely.

5. Load the sample by pipetting it below the surface of the sample loading solution in the loading funnel. Wash the sides of the empty sample tube with 20 µl neat TFA and add to the solution in the sample funnel. Rinse the pipet tip by pipetting the solution in the funnel into and out of the tip several times.

6. Load the sample onto the sample column using nitrogen pressure until all liquid has passed through the column. Allow nitrogen to pass through the column for several minutes to ensure complete drying of the column.

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7. Remove the top half of the sample cartridge from the loading station and reattach it to the bottom half of the sample cartridge. Insert the cartridge assembly into the sequencer. Check the levels of all needed items—e.g., sequencing reagents, HPLC solvents, argon, and printer paper—to ensure that sufficient quantities are available to complete the desired number of Edman cycles. Carefully select the appropriate sequencing programs for the samples to be analyzed and carry out the sequence run.

When multiple samples are to be analyzed, verify that the sequencer recognizes the presence of each cartridge before starting the first analysis. This is accomplished after all sample cartridges have been loaded and inserted onto the sequencer by manually selecting each sample cartridge position in turn from the protein sequencer menu.

At least one PTH standard or a PTH analyzer blank run followed by a PTH standard should be injected immediately prior to each sequence run to verify retention times and to determine calibration factors for each amino acid. The number of Edman cycles that should be run depends upon the characteristics of the sample and the purpose of the analysis. For most applications, it is recommended that peptides be sequenced through their C-terminus plus two cycles (to ensure that the entire sequence has been obtained and to assess background levels of amino acids (see Support Protocol 3). If the peptide's mass has been determined prior to the sequence run, the number of cycles to run can be estimated by: cycles = (peptide mass/100) + 3 (rounded up to next integer). For example, if the observed mass is 1644.5 Da, the sequencer can be set to complete 20 sample cycles. When intact proteins or large peptides are being analyzed and a maximum amount of sequence data is desired, set a number of cycles greater than the number that will be completed in an overnight run; the next morning review the data and continue the sequence until the decreasing signal-to-noise level limits sequence assignments (see Support Protocol 3). If the protein sequence is known and the purpose is to verify an intact N-terminal sequence or to define a cleavage site, 5 to 6 cycles should be sufficient. If the protein is to be searched against sequence databases to identify the protein, analysis of at least 20 cycles is recommended.

The Hewlett-Packard G1005A sequencer can be programmed to automatically analyze up to four samples in tandem. Therefore, care must be taken to ensure that sufficient reagents/solvents are available for the total cycles programmed and that the appropriate sequencing programs have been selected for the sample(s) to be analyzed.

SEQUENCING PVDF-BOUND SAMPLES ON A BIPHASIC CARTRIDGE SEQUENCER

BASIC PROTOCOL 4

PVDF-bound samples are prepared in a similar fashion regardless of the sequencer to be used (see Basic Protocol 2). PVDF samples sequenced in the biphasic cartridge sequencer are loaded to a reaction cartridge that has an empty top half rather than a top half packed with C18 resin like that used with liquid samples (see Fig. 11.10.2).

Materials

PVDF-bound sample(s) to be sequenced
Methanol in wash bottle
Source of argon or nitrogen

Reaction cartridge(s) for PVDF samples (Hewlett-Packard)
Glass gel plate
Stainless steel scalpel and stainless steel or Teflon-coated forceps
Bath sonicator

Additional reagents and equipment for sequencing on a biphasic cartridge sequencer (see Basic Protocol 3)

NOTE: Refer to the manufacturer's instructions for the Hewlett-Packard Model G1005A sequencing system at all steps.

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NOTE: Prepare solutions with Milli-Q water or equivalent taken directly from the purification unit. Water stored for any length of time in glass or plastic containers supports microbial and algal growth, which results in high amino acid background in early sequencer cycles.

1. Precondition the reaction cartridge (see Basic Protocol 3, step 1).
2. Using a scalpel, excise the desired stained protein band from a PVDF membrane, keeping the membrane on a clean, dry surface such as a glass gel plate and handling the membrane with a forceps.

   The glass plate, scalpel, and forceps should be thoroughly cleaned before use with Milli-Q water and methanol. Wear powder-free gloves rinsed in Milli-Q water. Do not touch the membrane or surfaces that contact the membrane with bare hands.

   Dry PVDF membranes are electrostatic and can be difficult to handle. The electrostatic nature of the membranes also attracts airborne contamination that invariably contains amino acid and protein contaminants. Special caution most be exercised to minimize contamination of the sample when working with small amounts of protein (<20 pmol).

   A total membrane area of up to 60 mm^2 can be loaded in the top half of the reaction cartridge.
3. While holding the membrane(s) with a stainless steel or Teflon-coated forceps, thoroughly rinse with a stream of methanol from a wash bottle. Immediately rinse the membranes with a stream of Milli-Q water delivered directly from the Milli-Q purification system.

   PVDF membranes can not be solvated directly with aqueous solutions and must initially be wetted in a strong organic solvent such as methanol. After the membranes are wetted with methanol in the above step, do not allow the membranes to dry out until drying is indicated in step 4.
4. Place the membrane(s) in a clean beaker containing 5 ml Milli-Q water and sonicate 5 min in a bath sonicator. Remove the membrane(s) with forceps, rinse with methanol, and dry under a gentle stream of argon or nitrogen.
5. Insert the membrane(s) in the top half of the sample cartridge.

   Because the opening is small (1-mm i.d.), membrane(s) can be cut into strips along their long axis and loaded as a stack or singly. An alternative to cutting strips is to fold the membrane along its longest axis with the protein side facing out. Load carefully so as not to damage the inside of the reaction cartridge chamber or the column-column joint within the sample cartridge assembly.
6. Reassemble the sample cartridge and insert the cartridge assembly into the sequencer.

   When multiple samples are to be analyzed, verify that the sequencer recognizes the presence of each cartridge before starting the first analysis (see Basic Protocol 3, step 7).
7. Check the levels of all needed items—e.g., sequencing reagents, HPLC solvents, argon, and printer paper—to ensure that sufficient quantities are available to complete the projected number of sequence cycles. Carefully select the appropriate sequencing programs for the samples to be analyzed and carry out the sequence run.

   At least one PTH standard or a PTH analyzer blank run followed by a PTH standard should be injected immediately prior to each sequence run to verify retention times and to determine calibration factors for each amino acid. The number of Edman cycles that should be run depends upon the purpose of the analysis. When intact proteins are sequenced and a maximum amount of sequence data is desired, set a number of cycles greater than the number that will be completed in an overnight run; the next morning review the data and continue the sequence until the decreasing signal-to-noise level limits sequence assign-

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ments (see Support Protocol 3). If the protein sequence is known and the purpose is to verify an intact N-terminal sequence or to define a cleavage site, 5 to 6 cycles should be sufficient. If the protein is to be searched against sequence databases to identify the protein, analysis of at least 20 cycles is recommended.

OPTIMIZING SEPARATION OF PTH AMINO ACIDS

SUPPORT PROTOCOL 1

Successful N-terminal sequencing is dependent on both the N-terminal sequencer and the in-line HPLC system that separates the PTH amino acids from each sequencer cycle. Both systems must be operating optimally to obtain a maximum amount of sequencing information, especially when analyzing samples in the low picomole range. The HPLC system should provide complete separation of all the commonly occurring PTH-derivative amino acids as well as sequencer reagent–related peaks. Also, retention times of all peaks should be highly consistent over the course of a sequence run. For high-sensitivity work (<10 pmol), baseline shifts must be minimal and reagent purity must be adequate so that labile amino acid derivatives are not destroyed.

The HPLC connected to the HP G1005A biphasic reaction column sequencer (see Basic Protocols 3 and 4) is a three-solvent system using preformulated solvents provided by the manufacturer. This HPLC system typically needs little or no adjustment in the parameters discussed below—e.g., gradient and solvents.

The following guidelines are for an HPLC system connected to the Perkin-Elmer Procise Model 491, 492, or 494 sequencer; however, the same separation system can also be used on older sequencers. A typical gradient program is shown in Table 11.10.4 and a summary of typical solvent and gradient adjustments is illustrated in Table 11.10.5.

Table 11.10.4 Gradient for Separation of PTH amino acids[a]

Step no.	Time (min)	%B
1	0.0	8
2	0.2	11
3	0.4	14
4	18.0	44
5	18.5	90
6	21.0	90

[a]Flow rate of 325 µl/min used throughout.

Table 11.10.5 Optimizing the Separation of PTH Amino Acids

Problem	Corrective action
Rise in baseline late in chromatogram	Add small aliquots of 1% acetone to buffer A
Negative baseline between DTT and Glu	Add small aliquots of 1.0 M KH_2PO_4 to buffer A
His too close to Ala or eluting after Ala	Increase Premix in buffer A
Arg too close to Tyr or eluting after Tyr	Increase Premix in buffer A
Ile/Lys not well separated (Lys early)	Decrease %B at 18-min point in gradient
Lys/Leu not well separated (Lys late)	Increase %B at 18-min point in gradient

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Additional Materials (also see Basic Protocol 1)

1% (v/v) acetone in Milli-Q water
1.0 M KH_2PO_4 in Milli-Q water
PTH Analyzer Standard mixture (standard mixture of PTH amino acids; ABI Biotechnology)
1-liter bottles and three-valve caps (Rainin)

NOTE: Prepare solutions with Milli-Q water or equivalent taken directly from the purification unit. Water stored for any length of time in glass or plastic containers supports microbial and algal growth, which results in high amino acid background in early sequencer cycles.

1. Prepare the "A solvent" by adding 15 ml Premix Buffer Concentrate to 1 liter of solvent A3.

   Unopened bottles of solvent A3 and the Premix Buffer Concentrate are stored at 4°C; partially used bottles of solvent A3 with additives can be stored at room temperature after displacing the air in the bottle with argon. Solvent A3 should be replaced after being in use for 7 to 10 days at room temperature. Peroxides will form with time in this solvent at room temperature and will adversely affect the yield of labile amino acids, especially lysine. The Premix Buffer Concentrate contains an ion-pairing additive that affects the peak shape and elution position of PTH-Arg, PTH-His, and the pyridylethyl derivative of cysteine. Addition of 15 ml Premix Buffer Concentrate to 1 liter A3 should position the peak for His in front of Ala and the peak for Arg in front of Tyr but after the dehydroalanine derivative of Ser.

2. Prepare the "B solvent" as follows. Dissolve the contents of one tube (500 nmol) of DMPTU in 1 ml solvent B2, vortex the tube, and allow to sit at least 15 min with additional vortexing every 5 min. Add the entire contents to 1 liter of solvent B2.

   Solvent B2 should resolve PTH-Trp from the typical Edman degradation product diphenylurea (DPU). Addition of 500 nmol DMPTU improves the recovery of PTH-Ile, PTH-Lys, and PTH-Leu, especially when analyzing <10 pmol. The rise in baseline late in the chromatogram that is caused by the addition of DMPTU may need to be adjusted with the addition of acetone to the A3 solvent (see step 6).

3. Transfer the freshly prepared solvents from steps 1 and 2 to two separate clean, dry 1-liter bottles, each sealed with a three-valve cap.

   The caps from Rainin are more robust than those supplied by the sequencer manufacturer and are easily replaced if leaking of the argon used to pressurize the bottles occurs.

4. Attach the bottle containing the A solvent to the A pump of the sequencer and the bottle containing the B solvent to the B pump. Purge the A and B pumps with the fresh solvents to eliminate any air bubbles in the pumps or solvent supply lines.

   The purge also replaces any old solvents in the pumps so that the first gradient is run entirely with the fresh solvents.

Optimize baseline

5. Repeatedly run the (blank) gradient illustrated in Table 11.10.4 using the "Run Gradient" program, each time adjusting the composition of the A solvent to render the baseline as flat as possible (steps 6, 7, and 8). Each time adjustments are made to the solvent composition, purge the pump with the adjusted solvent and run another blank gradient to observe the effects of the additions on the baseline.

6. Flatten late baseline rise by adding acetone in Milli-Q water to the A solvent.

   Small amounts of 1% acetone in 100-µl increments can be added to the A solvent to produce an absorbance match between the A and B solvents. This results in a flat baseline when a

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linear gradient is used. Typically, ~2 ml of 1% acetone is required per liter of A solvent to achieve this effect in a case where DMPTU is added to B solvent as described in step 2.

7. Correct early negative baseline slope by adding 1.0 M KH_2PO_4 to the A solvent.

   Frequently, a negative slope is observed in the beginning of the chromatogram between DTT peak and Glu (see Fig. 11.10.4). Addition of up to 100 µl of 1.0 M KH_2PO_4 in 10-µl increments will flatten the baseline in this area.

8. For high-sensitivity sequence analysis (<10 pmol), continue to adjust the A solvent as necessary with 1% acetone and 1.0 M KH_2PO_4 until the rise in baseline is no more than 0.1 mAU between 0 to 21 min.

Optimize positions of PTH amino acid peaks

9. After the baseline has been optimized, inject the PTH Analyzer Standard mixture by running the "PTH-Standards" program and evaluate the separation. If needed, repeat the run adjusting the composition of the A solvent to optimize the separation (steps 10 and 11). Each time adjustments are made to the solvent composition, purge the pump with the adjusted solvent and carry out another standard run to observe the effects of the additions on the chromatogram.

   The chromatogram illustrated in Figure 11.10.4 shows good separation of the common PTH amino acids and the Edman degradation byproducts DMPTU, DPTU, and DPU.

10. Add additional Premix Buffer Concentrate to the A solvent to move the His and Arg peaks earlier in the chromatogram if needed.

    Aging of the PTH analyzer column may necessitate increasing the amount of Premix Buffer Concentrate in order to maintain the position of His and Arg. Add Premix Buffer Concen-

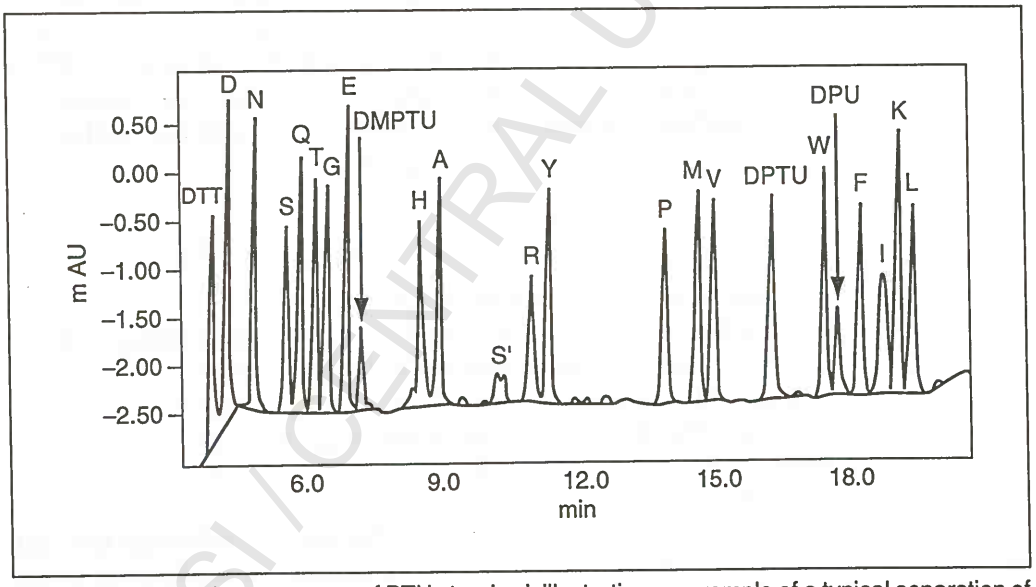

Figure 11.10.4 Chromatogram of PTH standard, illustrating an example of a typical separation of PTH amino acids and Edman degradation byproducts for the Perkin-Elmer Procise Models 491, 492, and 494 sequencers. The common amino acids recovered during the sequencing process are well separated, as are the normal byproducts of the Edman process: DMPTU (dimethylphenylthiourea), DPTU (diphenylthiourea), and DPU (diphenylurea). Unmodified cysteine is completely destroyed. Dithiothreitol (DTT) is typically present in the R4 reagent. S′ is an adduct between DTT and PTH-dehydroalanine, the latter of which is a degradation product of PTH-serine. Unmodified cysteine also forms S′, and can be distinguished from a serine residue because only the S′ peak increases in cycles containing unmodified cysteine. However, assigning cysteine based solely on this minor, often somewhat variable peak is risky. Abbreviations: mAU, milli–absorbance units; see Table A.1A.1 for one-letter amino acid abbreviations.

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trate to solvent A in 2-ml increments to position His before Ala and Arg before Tyr. The ability of Premix Buffer Concentrate to shift His and Arg retention times is lost if >25 ml are added to the A solvent. In this case, the A solvent can be prepared again as in step 1, but with the amount of Premix Buffer Concentrate added to a liter of A3 reduced to 12 ml, which should position His after Ala and Arg after Tyr. Alternatively, the PTH analyzer column can be replaced with a new one.

11. If further optimization of the separation is necessary for Ile, Lys, and Leu, reposition these peaks by making changes to the gradient as noted in Table 11.10.5.

Ideally, Lys should be centered between Ile and Leu. Consult the manufacturer's instructions if any other separation problems are observed.

SUPPORT PROTOCOL 2

INSTALLING AN INTERNAL HPLC RESTRICTER LOOP AND OPTIMIZING MAXIMUM INJECTION VOLUME ON OLDER SEQUENCERS

The Perkin-Elmer Procise Model 491, 492, and 494 sequencers monitor the volume of sample injected onto the in-line HPLC by using fluid sensors on the inlet and outlet of the sample loop. However, most older Perkin-Elmer/ABI Biotechnology sequencers have a 50-µl injection loop that results in injection and analysis of only ~42% of the total sample from the converter flask (~120 µl). Clogging of the external restricter also frequently occurs, which results in lost sequence data. The proportion of sample injected can be increased to ~85% and the risk of clogging the injector can be avoided by modifying the sample loop using a 100-µl loop with an internal restricter. Injecting a larger portion of the sample onto the HPLC increases sensitivity and is particularly helpful for high-sensitivity sequencing. Simply adding a larger loop would result in a very narrow time window for proper loop loading, which would result in variable injections due to inconsistent filling of the larger loop. The addition of an internal restricter avoids variable injections, as sample flow slows markedly before the loop is completely filled. The time window for loading the loop prior to injection is hence much longer and results in more consistent injections. Using the modified loop, a maximum amount of sample, ~102 µl out of 120 µl total or 85%, of the total volume is injected. In addition, the probability of missed injections resulting from a clogged restricter line is dramatically reduced because the internal restricter is back-flushed upon each injection.

Materials

Stainless steel tubing cutter
100-µl stainless steel rheodyne injection loop (Rainin)
Two restricter lines: 30-cm (length) × 0.007-in. (i.d.) stainless steel tubing with appropriate stainless steel nuts and ferrules (Upchurch)
Zero-dead-volume coupler (Upchurch)
Rheodyne injector on older Perkin-Elmer sequencer (Fig. 11.10.5)

Add modified injector loop

1. Using a stainless steel tubing cutter, cut the end fitting off one end of any commercially available rheodyne 100-µl stainless steel loop, immediately behind the ferrule (if in place on loop).

2. Connect the cut end of the 100-µl loop to one piece of restricter tubing using a zero-dead-volume coupler by inserting one piece of tubing halfway into the zero-dead-volume fitting and holding it in place while tightening the nut onto the ferrule. Insert the second piece of tubing as far as possible into the coupler and hold it firmly in place while tightening the connection.

Care should be taken to ensure that there is no gap between the two pieces of tubing in the zero-dead-volume connector.

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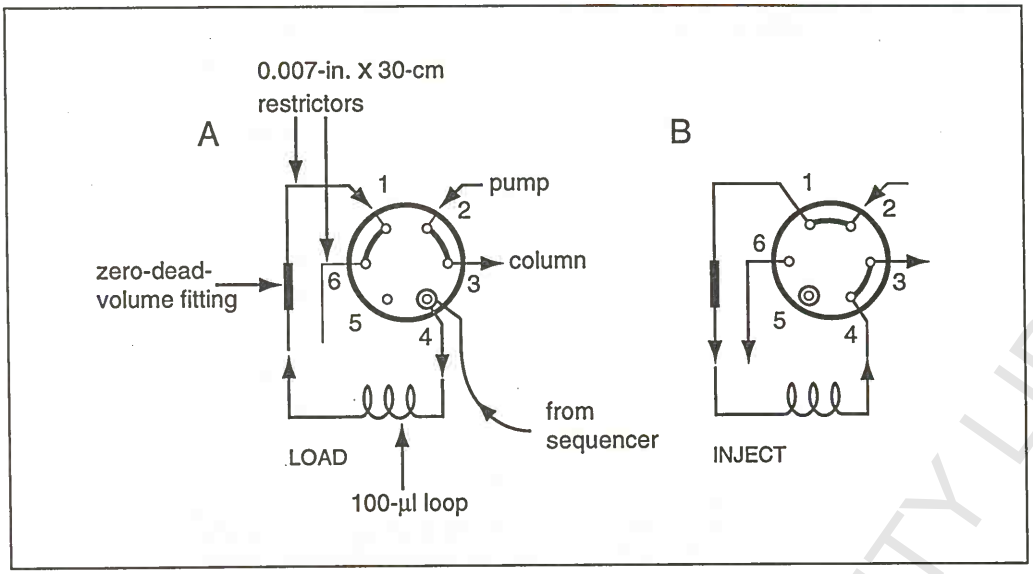

Figure 11.10.5 Illustration of an injector loop from an older model sequencing system, modified with an internal restricter. One end of the internal restricter is connected by a zero-dead-volume fitting to a 100-µl loop and the other end is connected to port 1 of the injector. The external restricter is connected to port 6 (overflow tube) and the two restricters aid in controlling the proper filling of the 100-µl loop/internal restricter assembly. As the sample is loaded (**A**), the restricters are encountered last and slow the speed of flow as the loop is nearly filled. When the sample is injected (**B**), the flow though the internal restricter is reversed. This reversal of flow on each injection prevents clogging of the restricter lines, which is normally the major cause of lost sequence data.

3. Connect the restricter end of the modified loop with port 1 of the injector valve and the full-bore end of the modified loop to port 4 (see Fig. 11.10.5).
4. Attach the second restricter line to port 6 of the rheodyne injector.
5. Flush injector loop with 100% acetonitrile in the Load position, switch injector to Inject position, run HPLC at typical flow rate, and check for leaks. Correct as required.

SEQUENCE DATA INTERPRETATION

SUPPORT PROTOCOL 3

After a sequencer run is completed, the next step is to assign a sequence by analyzing the data from all cycles. Although assigning some sequences can be straightforward—e.g., from a short, pure peptide at the 100-pmol level—accurate sequence assignment is often complex and usually requires an experienced scientist who is familiar with the recent sequencing performance history of the instrument used. Although Perkin-Elmer instruments have a software feature that automatically assigns sequences, an independent study that analyzed sequence assignment accuracy on an unknown sample showed that software assignments were much less accurate than those obtained by the average experienced sequencer operator using manual assignments (Yuksel et al., 1991). This same study showed that operators using the software-assigned sequence to assist them in their manual assignments achieved less accurate results than those operators who did not use software assignments at all. Care must be particularly exercised when interpreting the sequence of larger proteins, peptides containing contaminating (secondary) sequences, long peptide or protein sequence runs, and analyses where the signal is below the 10-pmol level.

Factors that must be considered when assigning sequence include the expected recovery of amino acids, the repetitive yield, signal carryover (lag) in subsequent cycles, and increases in background from nonspecific acid cleavage of peptide bonds. In addition,

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Table 11.10.6 Expected Recoveries of PTH Amino Acids[a]

Amino acid	Recovery (%)	
	Procise	HP G1005A
Arg	50-75	75-100
Asn	95[b]	95[b]
Gln	90[c]	90[c]
His	50	50-75
Ile	100	50[d]
Lys	75-90	50-100
Ser	30-50	10-20
Thr	50	25-40
Trp	10-40	20-70

[a]The common amino acids not listed are essentially quantitatively recovered on both sequencers (except cysteine which is completely destroyed during sequencing unless chemically modified to form a stable derivative).

[b]Asparagine is partially deamidated and detected as aspartic acid.

[c]Glutamine is partially deamidated (~10%) and detected as glutamic acid.

[d]Isoleucine on this system is resolved as its two isomers; one isomer migrates with phenylalanine.

accurate assignment of early cycles in a sequence can be problematic because of high amino acid background resulting from contamination of the sample (from many sources) prior to loading into the sequencer. In general, careful manual examination of the tabulated data from all cycles of the analysis, together with a comparison of the corresponding chromatograms, will produce the most accurate sequence assignment.

Expected Recoveries of PTH Amino Acids

Many PTH amino acids are essentially quantitatively recovered during the sequencing process, whereas others are partially destroyed or incompletely extracted (see Table 11.10.6). However, the recoveries of these problematic amino acids are usually fairly consistent for a given instrument, sequencer method, and set of reagents; they should normally be within the ranges indicated in Table 11.10.6. Lower-than-normal or variable recoveries are usually indicative either of chemical modification of the sample during isolation or problematic sequencer performance (resulting from hardware or reagent quality). When assigning sequences, the amino acid recoveries recently observed on the instrument used must be considered, especially when analyzing low-level or difficult sequence data sets.

Effects of Repetitive Yield on Number of Cycles Assigned

The background-corrected signal observed at each cycle of a sequence relative to the background-corrected signal observed in the previous cycle is the **repetitive yield.** Repetitive yield can be most easily calculated from the slope of the best-fit line when the logarithm of the net sequence signal observed (in pmol) is plotted versus cycle number. The size of the sequence signal always steadily decreases as a sequence run progresses, as a result of two factors. First, the efficiency of the Edman chemistry for each sequencer cycle is high but slightly less than 100%. A reasonable estimate for the overall efficiency of the reactions in each cycle when using an optimized sequencer and high-purity reagents

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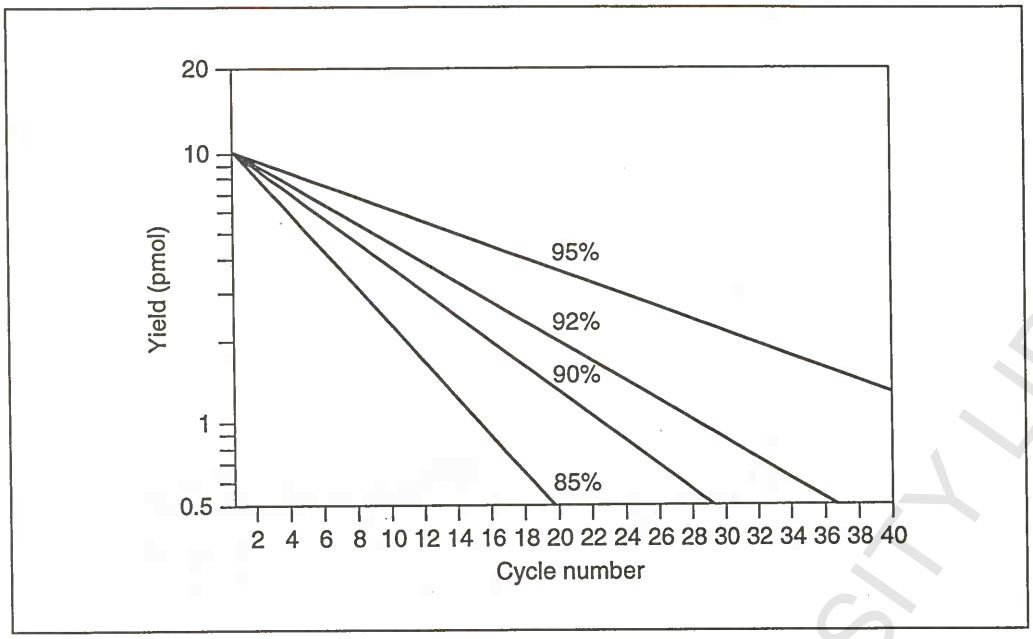

Figure 11.10.6 Effects of repetitive yield on number of cycles assigned. Theoretical amino acid yields for several different repetitive yields that are frequently encountered in automated sequence analysis illustrate the importance of this parameter on the amount of sequence that can be assigned. An initial yield of 10 pmol and a sequence detection limit of 0.5 pmol were used for this example.

would be ~96% to 98%. The second factor that decreases the yield is loss of sample from the sample support (sample washout). This second factor is very dependent upon the specific characteristics of individual samples, the type of sequencer support used, optimization of solvent deliveries in the sequencer, and related factors.

The relationship between repetitive yield and the amount of sequence that can be obtained is illustrated in Figure 11.10.6. In this example, an initial yield of 10 pmol and a sequencing detection threshold of 0.5 pmol is used. As shown, a repetitive yield of 95% would allow sequence assignment of >40 residues in this case, whereas lower repetitive yields decrease the maximum amount of sequence information that can be obtained. The observed repetitive yield for any sequencer run is related to both sample characteristics and sequencer operation. Optimization of all sequencer parameters to yield the highest possible repetitive yields, using an appropriate standard protein or peptide, will insure that the maximum amount of sequence can be assigned when sequencing experimental samples.

Carryover (Lag) Can Complicate Sequence Assignments in Later Cycles

Incomplete coupling and incomplete cleavage of the N-terminal residue during each sequencer cycle results in partial transfer of an amino acid signal into subsequent cycles. This effect is cumulative, so that carryover is usually but not always low in early cycles and progressively increases throughout the sequence. Cleavage at prolines is especially difficult, and an obvious jump in carryover at prolines is sometimes observed. The increase in carryover in later cycles and its influence on assigning sequences in later cycles is shown in Figure 11.10.7. The alanine signal in cycle 3 is very clear, with a small amount of carryover into cycle 4. The alanine signal at cycle 13 is much smaller, and the alanine value in cycle 14 is nearly equal to the value in cycle 13. The alanine in cycle 14 results from carryover and not an Ala-Ala sequence. Cycle 14 is a threonine, as indicated by the increase in signal of this residue in this cycle. Another alanine occurs in cycle 23 and the further increase in alanine signal in cycle 24 is due to a progressive further increase in

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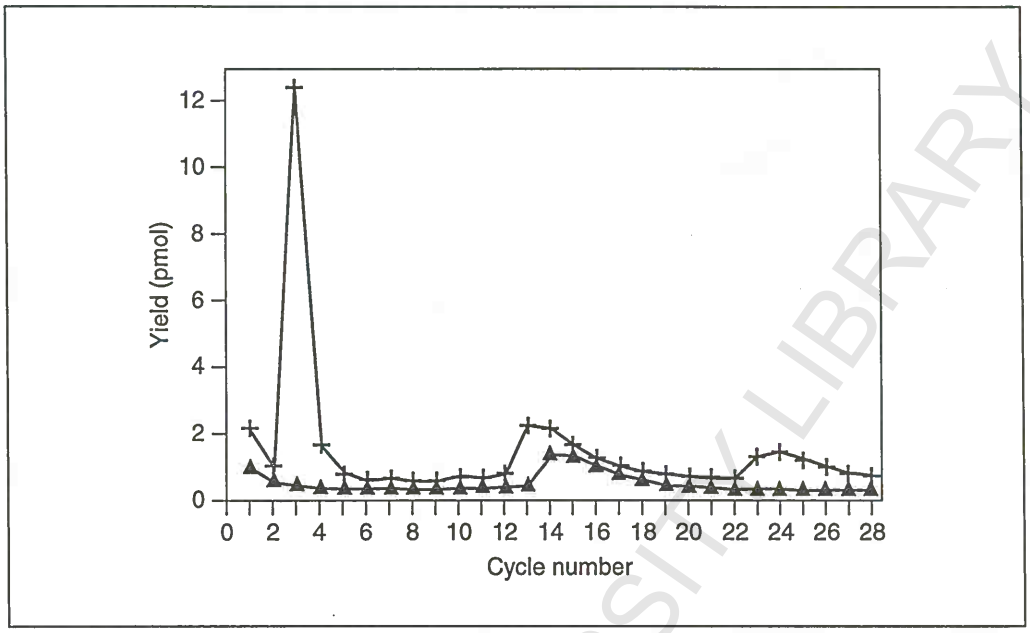

Figure 11.10.7 Yields of alanine and threonine from a sequence exhibiting somewhat higher-than-normal carryover. Uncorrected values of Ala (crosses) and Thr (triangles) in each cycle of a sequence are shown. The amount of carryover in this sequence was larger than the average for this instrument, but within the range of carryover seen with some samples. The high carryover in later cycles complicates sequence interpretation. This sequence contained Ala at cycles 3, 13, and 23, and Thr at cycle 14.

carryover. In this worse-than-average sequence, by cycles 13 and 14 carryover of the alanine and threonine signals, respectively, extend into the $n + 1$, $n + 2$, $n + 3$, and $n + 4$ cycles before the value of the amino acid returns to background levels.

Background in Early Sequencer Cycles

Assigning the first several residues in a sequence can often be difficult because of contaminants introduced during sample loading or from the sample itself, especially when the initial sequence signal is <10 pmol. These contaminants are primarily free amino acids and small peptides from such sources as airborne contamination, ungloved fingers, and buffers used in the purification. They result in a high level of multiple amino acids (background), especially glycine and serine, in the first one or more sequencer cycles.

The first four cycles from a sequence run using 10 pmol of β-lactoglobulin loaded to a GFF are shown in Figure 11.10.8A as an example of a very clean sample with minimal initial background. The first residue, leucine, as well as subsequent cycles can be easily assigned. The sequence assignment for the four cycles shown is LIVT (i.e., Leu-Ile-Val-Thr; see Table A.1A.1 for one-letter amino acid abbreviations). The sequence in Figure 11.10.8B is shown at the same sensitivity as the β-lactoglobulin sequence; however this experimental sample has substantial free amino acid and small-peptide contamination that results in a high background of several amino acids in early cycles. The multiple signals present in the first three cycles make assignment of sequence in these cycles difficult, but a clear glutamate (E) signal in cycle 4 and additional signals in subsequent cycles show that this protein is not blocked; hence a definitive sequence assignment from cycle 4 on was made. Very tentative assignments for cycles 2 and 3 were made based on knowledge of how rapidly background normally declined in analogous samples in this sequencer; however, the more conservative assignment for the cycles shown would be XXXE (where X represents an unassigned residue).

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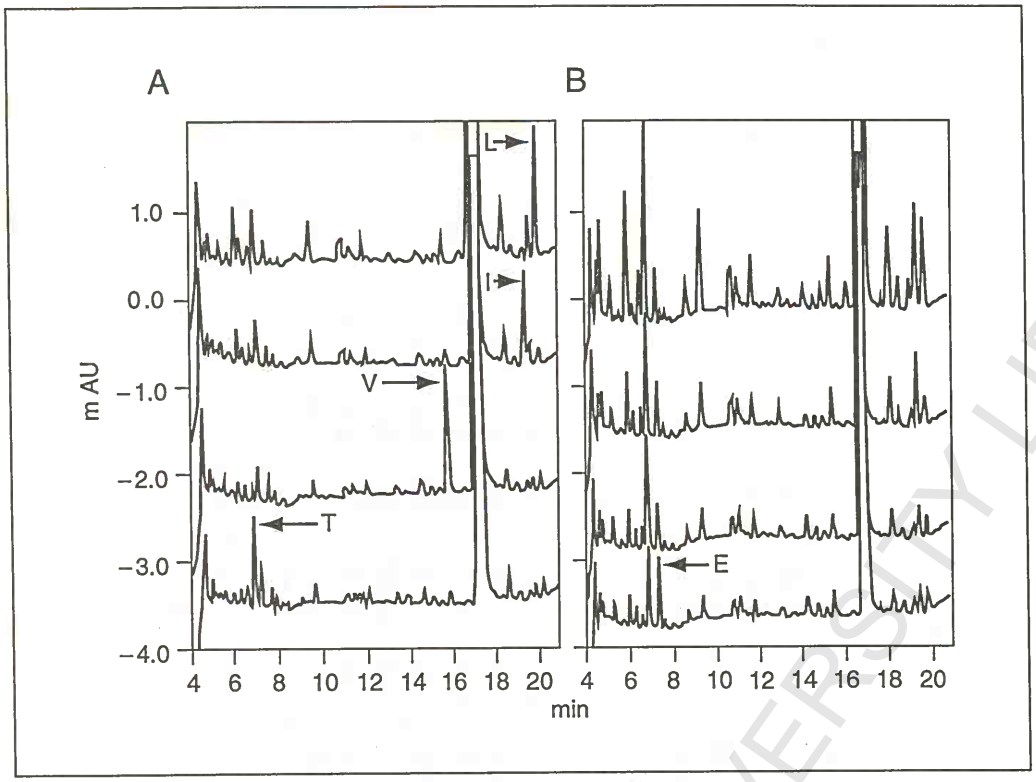

Figure 11.10.8 Chromatograms of the first four cycles from two different low-pmol-level sequences. **(A)** 10 pmol β-lactoglobulin were loaded onto a Procise 494. The first four residues can be clearly identified above a moderate initial background. **(B)** A tryptic peptide loaded to the same sequencer. In this case, the high background starting in cycle 1 interferes with sequence assignment of early cycles and the first unambiguous assignment that can be made is E, in cycle 4. Abbreviations: mAU, milli–absorbance units; see Table A.1A.1 for one-letter amino acid abbreviations.

Calculation of Background-Corrected Signals

In addition to early-cycle background from contaminants, most sequences exhibit a detectable level of most amino acids in most cycles of the sequence. The major source of this background is "nonspecific" acid cleavage of peptide bonds that free new internal sequences during the cleavage step of each sequencer cycle. Because there are fewer peptide bonds in peptides as compared with proteins, background is usually minimal for peptides, but it becomes increasingly higher for larger proteins as they have more peptide bonds to be randomly cleaved. The new internal N-termini start to produce many very low-level sequences in subsequent cycles. As the degree of cleavage is extremely low and somewhat random, a fairly uniform background results, which steadily increases throughout the sequence as more peptide bonds are cleaved at each cycle. Peptide bonds involving serine, threonine, and aspartic acid are the most labile; proteins or peptides that have a high content of these residues usually show higher background levels than other proteins of the same size. Table 11.10.7 illustrates data from 10 pmol of β-lactoglobulin loaded to a GFF and sequenced for 15 cycles with the Procise 494. The picomole values listed for each amino acid/cycle are the uncorrected or raw values automatically calculated versus the PTH standard (see Fig. 11.10.4) injected at the beginning of the run. Because β-lactoglobulin is a fairly small protein, there is detectable background for most amino acids; this increases steadily throughout the sequence run but it does not interfere with sequence assignment.

The accuracy of the reported values in this table should be verified by examining each chromatogram and correcting any problems such as incorrect peak identification or

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Table 11.10.7 PTH Amino Acid Yields (Uncorrected) from 15-Cycle Sequence Using 10 pmol β-lactoglobulin[a]

	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1	1.81	0.45	0.79	0.45	2.41	0.36	0.80	1.95	**[6.86]**	0.32	0.48	0.41	1.03	0.48	3.21[d]	0.93	0.83	0.10	0.65
2	0.92	0.47	0.60	0.34	1.91	0.15	**[7.76]**	0.86	0.75	0.15	0.45	0.48	0.59	0.62	1.73	0.45	0.46	0.00	0.44
3	0.70	1.20[b]	0.57	0.28	1.54	0.16	0.76	0.58	0.66	0.13	0.42	0.60	0.51	0.47	0.94	0.37	**[5.95]**	0.00	0.42
4	0.66	0.78	0.59	0.24	1.52	0.20	0.53	0.53	0.67	0.13	0.28	0.54	0.61	0.66	0.79	**[4.00]**	0.60	0.13	0.43
5	0.76	0.98	1.15[c]	0.25	1.49	0.16	0.51	0.52	0.83	0.10	0.38	0.56	**[5.24]**	0.60	0.67	0.48	0.47	0.00	0.42
6	0.78	0.56	0.71	0.17	1.48	0.11	0.41	0.42	1.03	0.13	0.38	0.60	0.91	0.72	0.65	**[3.47]**	0.58	0.00	0.50
7	0.74	0.88	0.76	0.30	1.53	0.19	0.44	0.43	1.02	**[4.36]**	0.38	0.56	0.64	0.68	0.64	0.53	0.55	0.00	0.50
8	0.77	0.63	0.80	0.26	1.68	0.16	0.30	**[3.59]**	0.98	0.51	0.38	0.49	0.66	0.66	0.72	0.40	0.52	0.09	0.52
9	0.74	0.66	0.76	0.34	**[5.05]**	0.14	0.60	0.90	1.02	0.23	0.37	0.47	0.69	0.70	0.67	0.35	0.52	0.03	0.55
10	0.84	0.62	0.75	0.36	2.05	0.09	0.53	0.55	**[4.99]**	0.20	0.35	0.46	0.69	0.80	0.72	0.32	0.60	0.04	0.56
11	0.87	**[3.66]**	0.75	0.37	1.83	0.09	0.00[b]	0.51	1.61	0.26	0.35	0.45	0.68	0.69	0.78	0.27	0.61	0.00	0.59
12	0.88	0.99	0.76	0.37	1.83	0.13	**[4.85]**	0.56	1.29	0.26	0.26	0.42	0.67	0.73	0.77	0.27	0.62	0.00	0.54
13	0.85	0.72	1.16[c]	0.35	1.87	0.14	1.10	0.49	1.26	0.24	0.37	0.45	**[4.07]**	0.68	0.73	0.26	0.58	0.05	0.62
14	0.87	0.67	0.83	0.42	1.89	0.14	0.74	**[2.97]**	1.21	0.23	0.39	0.22	1.15	0.79	0.81	0.30	0.57	0.03	0.58
15	0.90	0.64	0.76	0.41	1.92	0.08	0.67	0.94	1.27	0.23	0.42	0.51	0.80	0.79	0.79	0.32	**[3.71]**	0.04	0.57

[a]The assigned sequence is indicated by brackets.

[b]Value incorrect due to incorrect baseline.

[c]Glu value has increased due to partial deamidation of Gln in cycle.

[d]Ser contamination is common in cycle 1.

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inconsistent peak integration. In some cases, it may be necessary to reintegrate and reprint the entire sequence run prior to manually computing the net or background-corrected amino acid yields of assigned residues in the sequence. After the accuracy of reported values is verified, an appropriate background level can be estimated for each observed sequence signal and subtracted from the sequence signal observed in cycle *n*. In general, the most appropriate background value is the amount observed in the cycle before the sequence signal ($n - 1$). For example, in Table 11.10.7 the background-corrected value for Val in cycle 3 would be 5.5 pmol (5.95 – 0.46 = 5.49). An alternative to evaluating a table of raw data values as shown in Table 11.10.7 is to plot the values for each residue versus cycle number—i.e., the type of plots shown in Figure 11.10.7 for an unrelated sequence.

Careful evaluation of net sequence yields for each cycle is especially important for complex sequence data sets and can be useful in identifying potential errors in initial sequence assignments. Especially when one or more lower-level (secondary) sequences are present in addition to a major (primary) sequence, evaluation of sequence yields minimizes the risk of confusing signals from the secondary sequence with positions in the primary sequence, where labile or post-translationally modified residues might occur.

Sequence Assignment

The simplest data set from a sequenced sample is one signal per cycle, as illustrated by the data set in Table 11.10.7. In this case the sequence is easily assigned—e.g., LIVTQ TMKGL DIQKV. Assignments are typically reported using the following conventions: uppercase letters for positive calls (confidence level should be >99%); lowercase letter or letter enclosed by parentheses for a tentative call (confidence level between 50% and 99%); and X for no assignment possible.

When two or more signals are present in each cycle from the sample sequenced and the sequence is unknown, assignment becomes more challenging. A single major sequence in the presence of one or more sequences at much lower levels (less than a third the major sequence level) can usually be assigned with substantial confidence. Sorting of multiple sequences must rely heavily on quantitative yields and must consider corrections for residues not recovered quantitatively (Table 11.10.6). In some cases, a minor sequence can also be assigned, but assignment of some residues in this sequence may be complicated by interference that is due to carryover of the major sequence.

Mass Analysis Can Aid Sequence Assignments

As briefly discussed in Strategic Planning, obtaining a mass for HPLC-purified peptides by MALDI mass spectrometry (*UNITS 11.6 & 16.2*) can aid in assigning sequences. When the entire sequence is assigned by Edman sequencing, comparing the calculated and observed masses provides a useful check that the correct sequence assignment has been made. Similarly, when one or two residues have been assigned tentatively and the remainder of the peptide sequence has been positively assigned, the confidence level of the tentative calls can often be increased or decreased by considering the observed mass. Finally, when a single residue cannot be assigned and the remainder of the sequence has been positively assigned, the difference between the observed and calculated masses will yield the mass of the unassigned residue, which frequently suggests a likely assignment for this position. Examples where this approach is useful include: an X (unassignable residue) in the first cycle resulting from initial background, a missing last residue in a tryptic peptide resulting from washout of the last residue, an unmodified cysteine, or a post-translationally modified residue not detected by Edman sequencing. Mass analysis of peptides is therefore a very valuable complementary method when used in conjunction with Edman

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sequencing. However, caution must be exercised when using mass analysis to assign residues where no or multiple Edman signals are present. In some cases, more than one possible interpretation of tentative or unassigned residues may fit within the error limits of the mass analysis, which can be up to ±0.1% or more, depending upon the calibration method and instrument used.

SUPPORT PROTOCOL 4

OPTIMIZING SEQUENCER PERFORMANCE

Optimal performance of a protein sequencer depends on careful and frequent evaluation of multiple parameters including proper instrument delivery of solvents and reagents, quality of sequencer reagents and solvents, sequencer performance, and yields of non-quantitatively recovered amino acids when analyzing appropriate peptide and protein standards (Tempst and Riviere, 1989; Atherton et al., 1993; Tempst et al., 1994). For additional information on problems in sequencer performance and their solutions, see Troubleshooting.

Sequencer Standards

Commonly used sequencing standards include: human serum albumin (HSA; typically used for the Hewlett-Packard G1005A sequencer); β-lactoglobulin (typically used for the Procise 491, 492, 494, and other Perkin-Elmer sequencers). Horse apomyoglobin is also sometimes used as a protein standard. Peptide standards suitable for either sequencer include melittin or the sequencing test peptide from Sigma. A sequence of a standard sample should be evaluated at least once for every 10 to 20 experimental samples sequenced, to detect noncatastrophic sequence-performance problems. The standard sample should be analyzed at a level similar to the average levels of experimental samples that are being analyzed—e.g., 5, 10, or 50 pmol.

Standard protein or peptide solutions should be prepared in adequate quantity for multiple analyses, then quantified by amino acid analysis, divided into multiple aliquots, and stored at –20°C. A fresh aliquot can be used for every third standard run to minimize possible variation resulting from degradation or contamination of the aliquot during handling. Results from all sequences for each type of standard can be listed in a spreadsheet-format database that includes such items as background corrected yield for each cycle, repetitive yield, and carryover at a specific reference cycle. Sequences that exhibit optimal initial yields, maximum repetitive yields, low carryover, and good recoveries of all amino acids can then be used as reference point for evaluating sequencer performance in future sequence runs.

Proper Delivery of Solvents and Reagents During Sequencer Cycles

Each manufacturer publishes guidelines for evaluating and adjusting deliveries of all solvents and reagents used in the sequencing process for their respective sequencer. Direct observation should be made of all solvent and reagent deliveries for a complete Edman cycle (both reaction and conversion portions of the cycle) at least once every three to six sequencer runs; these should then be compared to the manufacturers recommendations. These observations should be kept in a log book containing a record of pertinent information corresponding to each sequence run. A sample log sheet for this purpose is shown in Figure 11.10.9. These records are useful for identifying trends, such as a gradual reduction in wash solvent volume delivery, before they become problematic to the point where sequencer performance is significantly affected. Significant adjustments to such parameters as delivery volumes and pressures should be evaluated by sequencing an appropriate standard to verify good sequencer performance prior to sequencing experimental samples.

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Quality of Solvents and Reagents

All solvents and reagents should be marked with the date of receipt prior to being stored according to the manufacturer's recommendations. Solvents and reagents should be free of any kind of particulate matter, haze, or cloudiness. The lot numbers should be changed in the sequencer-bottle menus whenever a bottle is refilled or replaced on the sequencer. The actual date that the bottle is placed on the sequencer should also be written on the label of the bottle and the appropriate information should be recorded in the sequencer log book (see Fig. 11.10.9). When analyzing samples at the <10-pmol level, many changes in sequencer performance that are not due to hardware problems can quickly be correlated

PE/ABI Sequencer Log

Seq. #_____
Date/operator_____
Program_____
Type_____
Cycles_____
Cart. pos._____

Sample name_____
PI/Submitter/Institution_____
pmol loaded_____ M.W._____

Cartridge leak test
psi set_____
psi after test_____

HPLC
A3_____
Pre mix #_____
Amt. added_____
B2_____
DMPTU lot #_____
Amt. added_____

Filter prep.
Biobrene lot #_____
Filter lot #_____
Cycles_____
Prog._____
Date_____ Pos._____

	Lot #	Description	Set press	Observations
S2B		ethyl acetate	1.7	
S3		*n*-butyl chloride	1.7	
S4B		20% acetonitrile in water	3.5	
S1		*n*-heptane	1.7	
R2B		*n*-methylpiperidine/water/methanol	1.0	
X3		100% methanol (in-house)	3.5	
R5		Standard	2.5	
R4A		25% TFA in water	3.5	
R3		TFA (neat)	1.5	
R1		5% PITC in heptane	2.5	
X2		100% methanol (in house)	2.5	
X1		1:1 methanol in water (in-house)	2.5	

	Tank	Set
Argon		

_____
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_____
_____
_____

Figure 11.10.9 A sample log sheet for recording critical observations for each sequence. This is for documenting reagent lot numbers, gas pressures, and observations of reagent/solvent deliveries, which facilitates troubleshooting when sequencer performance problems are encountered. If a log sheet is completed for each sequence, it can also serve as a checklist to ensure that adequate reagents, solvents, gases, and other necessary items are available for the entire sequencer run.

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with a lot change for a specific sequencer reagent by careful tracking of reagent lot numbers and installation dates.

In general, dramatic changes in sequencer performance are related to hardware failures or a marked decrease in the quality of the most recently changed solvent or reagent.

COMMENTARY

Background Information

Most protein sequencing projects fall into one of two groups: (1) partial sequence analysis of one or more proteins isolated from natural sources that correlate with a biological process of interest, or (2) analysis of recombinant proteins or fragments of these proteins as part of structure-function studies or verification of structural integrity for pharmaceutical applications. The chemical reactions used in modern sequencers involve the cyclic removal of the N-terminal residue from a protein or peptide after modification with phenylisothiocyanate (PITC). This basic chemistry remains largely unchanged since it was first described in 1949 by Pehr Edman (Edman, 1949). However, the amount of protein required for routine sequence analysis has steadily declined from micromole quantities using early automated sequencers (Edman and Begg, 1967) to the current 10- to 100-pmol levels (Yuksel et al., 1991; Crimmins et al., 1992). Although most sequencing laboratories can effectively sequence samples at the >100-pmol level, substantial optimization of instrumentation and associated methods must be invested to ensure that a sequencing laboratory can routinely obtain sequence data at the 10- to 100-pmol level. Only a small proportion of sequencing laboratories have optimized their methods sufficiently to permit fairly routine high-sensitivity sequence analysis at the 1- to 10-pmol level. Hence, the selection of an appropriate sequencing facility can be an important consideration for projects where the amount of sample is limited. The capacity to sequence samples at maximum sensitivity appears to be largely a function of the rigor that is devoted to instrument optimization, monitoring, and maintenance—rather than of differences in specific instrumentation or methods used (Tempst and Riviere, 1989; Yuksel et al., 1991; Crimmins et al., 1992).

In general, N-terminal sequence analyses of intact proteins and large peptide fragments are most effectively performed by electroblotting the sample from an appropriate one- or two-dimensional gel onto a high-retention PVDF membrane (*UNIT 10.7*). Electrotransfer efficiencies of 50% to 80% can usually be obtained for most proteins, and total losses including artifactual blocking of free N-terminals and transfer losses are usually <35% even at the <10-pmol level when appropriate precautions are taken (Mozdzanowski and Speicher, 1992; Speicher, 1994). Substantial N-terminal sequence information can be obtained from an electroblotted protein when as little as 2 to 5 pmol of protein is present on the blot and extensive blocking of the N-terminus has been avoided during sample preparation. When 2 pmol of a desired unblocked protein is electroblotted onto a PVDF membrane, the initial signal observed in the sequencer is expected to be between 0.4 and 1.4 pmol. As noted above, sequence analysis at this level is currently feasible, but certainly not routine. In some cases, it may be preferable to sequence a soluble, highly purified protein that is already in a solution compatible with direct sequence analysis (see Basic Protocol 1 or 3) instead of using SDS gels and electroblotting.

Because 50% to 80% of all proteins isolated from natural sources are physiologically blocked (Brown and Roberts, 1976), the current preferred strategy for obtaining partial sequence data from an unknown protein is to immediately attempt internal sequencing rather than N-terminal sequencing of the intact protein. This approach involves four sequential steps: in situ digestion with a specific protease such as trypsin either in the gel or on a PVDF membrane, microbore or narrow-bore reversed-phase HPLC separation of the tryptic peptides, MALDI mass analysis of selected peak fractions, and finally N-terminal sequencing of one or more of the largest peptides as indicated by the mass analysis.

Over the past several years, protein identification by mass fingerprinting has emerged as an increasingly useful alternative approach to Edman sequencing of unknown proteins. In this method, peptides from in-gel digestion of a protein band using a specific protease such as trypsin are analyzed as a mixture using MALDI mass spectrometry (*UNITS 11.6 & 16.2*). These multiple peptide masses are then searched against a protein database that has been transformed from a sequence database to a database

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Table 11.10.8 Troubleshooting and Optimizing Sequencer Performance[a,b]

Problem	Possible cause(s)	Suggested solution(s)
No sequence and no increase in background in later cycles	Insufficient sample	Load larger amount of sample and try to quantify amount loaded
No sequence; background does increase in later cycles	Sample is N-terminally blocked	Cleave protein using trypsin and sequence internal peptides and/or evaluate isolation procedure for conditions that might block N-terminus
No sequence; DMPTU, DPTU, and DPU peaks present	R4 bottle empty or reagent not delivered	Replace with full bottle of R4 and/or check delivery
No sequence and no injection solvent front	Extraction solvent bottle empty or solvent not delivered; injector clogged or not working	Replace extraction solvent with full bottle; check delivery of extraction solvent; fix injector
High DMPTU, DPTU, DPU	Poor washing of sample after coupling; poor R4 drydowns	Observe washes and optimize per manufacturer's recommendations; observe dry times and adjust as needed
PTC analogs of PTH amino acids present	Poor R4 drydowns	Observe dry times and adjust as needed
Low PTH amino acid yields in all cycles	Insufficient transfer of ATZs from sample support	Optimize extractions
High background in initial cycles	Sample excessively contaminated	Clean up sample, buffers, buffer water source, etc.
Low initial background increases too rapidly	Acid cleavage time is excessive	Reduce delivery of TFA (R3)
High lag, low RY, low DMPTU, DPTU, DPU	R2 depleted or too old; R2 delivery too short	Replace R2; check R2 delivery and adjust if needed
High lag after Pro	Typical after Pro residue	Can increase R3 reaction time for Pro-rich samples (but will increase background)
Low initial yield	R1 bottle empty or poor delivery; R1 too old; argon gas contaminated	Replace R1 and/or check delivery; replace if >30 days old; replace argon tank with higher-purity gas
Large peak early in chromatogram (air injected onto HPLC column)	Low delivery of S4; injection loop over/under filled; clogged flask pickup tube; S4 clinging to flask walls	Check S4 delivery and adjust if needed; adjust loop fill time; check values and sensors; clean pickup tube or replace; clean or replace flask

[a]If dramatic changes in delivery times are observed or if delivery is variable, possible hardware problems should be considered instead of simply adjusting delivery times for solvents and reagents.

[b]Abbreviations: ATZ, 2-anilino-5-thiazolinone; DMPTU, dimethylphenylthiourea; DPTU, diphenylthiourea; DPU, diphenylurea; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin; RY, repetitive yield; TFA, trifluoroacetic acid.

of tryptic peptide masses (Henzel et al., 1993; Mann et al., 1993; Pappin et al., 1993; Yates et al., 1993). The validity of protein identification by this method is dependent upon multiple factors including the accuracy of the masses used for the search. The stringency of the comparison can be increased dramatically if partial sequence information from tandem mass spectrometry methods (MS/MS; *UNIT16.2*) are used in addition to the tryptic peptide masses, either using post-source decay on a reflectron MALDI mass spectrometer or using an electrospray quadruple mass spectrometer (triple quadruple or ion trap). Of course, if the specific protein sequence from the species being studied is not in the database, a reliable match is unlikely. However, as the sequence databases are rapidly expanding to include complete genomes of multiple species, the utility of this approach will continue to rapidly expand. Most importantly, this approach has the potential to reliably identify proteins by mass matching with limited se-

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Table 11.10.9 Troubleshooting and Optimizing HPLC Performance[a,b]

Problem	Possible cause(s)	Suggested solution(s)
No peaks and no changes in baseline	HPLC pumps not running; detector lamp not on	Evaluate and correct HPLC problem(s); turn on lamp or replace if needed
Retention times for all PTH amino acids are variable	HPLC pump seals worn; A or B pump delivery erratic	Replace pump seals; identify problem pump and repair or replace
Retention times for early PTH amino acids are variable	Insufficient reequilibration prior to injection	Increase reequilibration time
PTH amino acid peaks become broader with time	Contaminants on guard column; PTH column aging	Replace guard column at least every 30 days; replace PTH column
Noisy baseline; erratic small sharp spikes in baseline	HPLC lamp aging	Replace lamp

[a]Also see Table 11.10.5 for baseline adjustments and optimization of PTH amino acid separation.

[b]Abbreviations: HPLC, high-performance liquid chromatography, PTH, phenylthiohydantoin.

quence analysis by MS/MS methods at one to two orders of magnitude higher sensitivity (100 to 1000 femtomoles of protein) than the current maximum sensitivity for internal sequencing (10 to 20 pmol) using the Edman sequencing approach described in this unit (Shevchenko et al., 1996).

Critical Parameters

When isolating proteins from natural sources, the most difficult part of the project is usually the isolation of a sufficient amount of the protein of interest in a form that is free of contaminating proteins, amino acids, and incompatible inorganic compounds. Purifying relatively low-abundance proteins from natural sources is particularly challenging. In such situations it is advisable to keep the purification scheme to the fewest possible steps and to use either one-dimensional SDS gels or two-dimensional gels for the final purification step. Regardless of the abundance of the target protein or purification method, the vast majority of proteins currently analyzed in sequencing facilities are separated by SDS-PAGE prior to either N-terminal sequence analysis or internal sequencing. Detailed guidelines for sample isolation are described above (see Strategic Planning) and should be carefully followed.

Troubleshooting

The first step in troubleshooting sequencer problems is to distinguish between sequence analysis problems and problems arising from the sample—i.e., sample heterogeneity, blocked N-terminal sequence, or insufficient sample loaded into the sequencer. Suspected sequence analysis problems can be readily evaluated by immediately analyzing an appropriate standard peptide.

Table 11.10.8 lists some of the more common problems encountered in sequencer operation and optimization; Table 11.10.9 lists common problems for the HPLC analyzer. In general, dramatic changes in sequencer performance are related to hardware failures or a marked decrease in the quality of the most recently changed solvent or reagent.

Anticipated Results

When the N-terminus of the protein or peptide loaded to the sequencer is not blocked, an initial sequence yield should be observed equal to ~20% to 80% of the amount of sample loaded into the sequencer. The first several cycles of any sequence may have signals from multiple amino acids, which can complicate making positive identifications for these cycles; however, tentative assignments can often be confirmed if the mass of the peptide has been determined prior to sequence analysis. The high initial background, if observed, should disappear by cycle 2 or 3 unless contamination is extremely severe. Some peptide and protein samples will contain minor contaminating sequences, and these secondary sequences can often be distinguished from the major sequence if a large quantitative difference exists. However, caution must be exercised in this case, as the repetitive yields of the major and minor sequences may be different because of differing degrees of sample washout. For example, a short major peptide may exhibit a lower repetitive yield than a minor longer peptide, and

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similar signals might be observed near the end of the major sequence, resulting in potential misassignment to the major sequence of subsequent residues in the minor sequence. When homogeneous peptides are being sequenced, the entire sequence can usually be assigned unless the peptide exhibits unusually high washout. When proteins or large peptides are being sequenced, several factors can limit the amount of sequence that can be obtained, including repetitive yield, progressive increase in carryover as the sequence proceeds, and increased background from nonspecific acid cleavage. Typically, most proteins exhibit high repetitive yields (>92%); hence repetitive yield is rarely the most limiting factor when sequencing proteins except when the sequencer is not optimized or the initial sequence yield is near the detection limit (usually 0.1 to 1 pmol). As a general rule, when sequencing proteins >100 kDa, increased background is likely to be the most important limiting factor. For proteins <100 kDa, the limiting factor is usually the rising carryover.

Time Considerations

The time required for preparation of the sequencer and HPLC prior to loading the samples can range from ~5 min (if sufficient sequencer reagents/solvents and HPLC solvents are on the instrument for the projected number of cycles) to up to 2 hr when multiple reagent replacements are needed. Loading PVDF-bound samples to the Procise sequencer requires ~15 min, which includes excising the sample from the membrane(s), washing it, drying it, and loading it into a clean Blott cartridge. Loading PVDF-bound samples into the Hewlett-Packard biphasic cartridge sequencer requires an additional 20 min, as the sample cartridge must be precycled on the sequencer. Loading liquid samples onto the Hewlett-Packard biphasic cartridge sequencer takes ~20 to 40 min for precycling the sample cartridge while simultaneously cleaning the sample loading funnel, followed by 20 min to load the sample onto the sample cartridge. Loading liquid samples onto the Procise or other sequencers using a glass fiber filter (GFF) sample support requires the most time. It takes ~15 min to load Polybrene on a GFF and set up two filter precycles that take ~35 min each to precondition the GFF on the sequencer prior to sample application. The time required for sample application will vary widely depending upon the volume to be loaded. It takes ~5 min to apply and dry 15 µl of sample on a preconditioned GFF. Loading large volumes using repetitive application can be quite time-consuming; for example, it would take about 1 hr to load 150 µl in 15-µl aliquots.

After the run is complete, the time required for analyzing a sequence is related to the length of the sequence and the complexity of the data. A straightforward 20-cycle peptide sequence may require as little as 30 min to analyze. In contrast a very complex 20-cycle multiple-sequence data set with integration problems may require at least 2 hr for careful data analysis. It is also advisable for two experienced sequencer operators to independently assign very complex sequences, to improve the accuracy of the assignments.

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POST-TRANSLATIONAL MODIFICATION: GLYCOSYLATION

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CHAPTER 12
Post-Translational Modification: Glycosylation

INTRODUCTION

The following units provide a comprehensive description of a widespread protein modification: glycosylation. The overview (*UNIT 12.1*) summarizes present knowledge of the type of carbohydrate modifications that occur on proteins, along with aspects of their biosynthesis. The protocols allow the investigator to tailor the analysis of the protein studied to different levels of sophistication. Metabolic labeling experiments (*UNIT 12.2*) in the presence of inhibitors (*UNIT 12.3*) can be used to determine the presence of protein-bound carbohydrates and to classify them as to general structure, especially when these methods are used in conjunction with enzymatic digestions (*UNIT 12.4*). An entirely distinct type of modification is the attachment of glycophospholipid anchors; methods for analyzing such modifications are described in *UNIT 12.5*. The protocols presented here should allow investigators confronted with the characterization of glycoproteins to carry the analysis to the point where the field of protein chemistry proper merges with that of glycobiology.

Hidde L. Ploegh

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Overview of Glycoconjugate Analysis

The modern revolution in molecular biology has been driven to a large extent by advances in methods for analysis and manipulation of DNA, RNA, and proteins. Although oligosaccharides (sugar chains) are also major macromolecules of the typical cell, they did not initially share in this molecular revolution. The reasons for this are to a large extent technical. Whereas DNA, RNA, and proteins are linear polymers that can usually be directly sequenced, oligosaccharides show substantially more complexity, having branching and anomeric configurations (α and β linkages). Thus, whereas three amino acids or nucleotides can be combined into six possible sequences, three hexose monosaccharides can theoretically generate 1056 possible oligosaccharides. Also, the syntheses of DNA, RNA, and proteins are template-driven, and the sequence of one can generally be predicted from that of another. In contrast, the biosynthesis of oligosaccharides, termed glycosylation, is extremely complex, is not template-driven, varies among different cell types, and cannot be easily predicted from simple rules.

It is clear that oligosaccharides have important, albeit varied, effects upon the biosynthesis, folding, solubility, stability, subcellular trafficking, turnover, and half-life of the molecules to which they are attached. These are matters of great importance to the cell biologist, protein chemist, biotechnologist, and pharmacologist. On the other hand, the successful growth of several glycosylation mutants as permanent tissue culture cell lines indicates that the precise structure of many oligosaccharides is not critical for the growth and viability of a single cell in the protected environment of the culture dish. Thus, until recently, it was possible for many researchers working with in vitro single-cell systems to ignore the existence of oligosaccharides. However, with the increasing emphasis on studying cell-cell interactions in normal development, tissue morphogenesis, immune reactions, and pathological conditions such as cancer and inflammation, the study of oligosaccharide structure and biosynthesis has become very important.

The term "glycobiology" has found acceptance for denoting studies of the biology of glycoconjugates in both simple and complex systems. Many technical advances have occurred in the analysis of oligosaccharides, making it now feasible to study them in detail. These advances include the development of sensitive and specific assays and the availability of numerous purified enzymes (glycosidases) with high degrees of specificity.

In spite of all these recent advances, many analytical techniques in glycobiology have remained in the domain of the few laboratories that specialize in the study of oligosaccharides. Likewise, published compendia of carbohydrate methods are designed mainly for use by experts. This chapter attempts to place some of this technology within easy reach of any laboratory with basic capabilities in biochemistry and molecular biology. The techniques described here include modern versions of time-honored methods and recently developed methods, both of which have widespread applications. However, it is important to emphasize that the protocols presented here are by no means comprehensive. Rather, they serve as a starting point for the uninitiated scientist who wishes to explore the structure, biosynthesis, and biology of oligosaccharide chains. In most cases, further analysis using more sophisticated techniques will be required to obtain final and definitive results. Nonetheless, armed with results obtained using the techniques described here, the average researcher can make intelligent decisions about the need for such further analyses.

The appearance of many commercial kits for analysis of glycoconjugates is another sign that the technology has arrived and that many laboratories have developed an interest in glycobiology. It is worth noting that although some of these kits are designed to simplify the use of well-established techniques, others employ methodologies that have been newly developed by the companies themselves. Experience with the latter methodologies in academic scientific laboratories may be limited, and the techniques in question may therefore not be represented in this chapter. However, although the admonition *caveat emptor* is appropriate, some kits may well become useful adjuncts to the methods presented here.

Different types of glycosylation are perhaps best defined by the nature of the linkage region of the oligosaccharide to a lipid or protein (Fig. 12.1.1). Although the linkage regions of these molecules are unique, the sugar chains frequently tend to share common types of outer sequences. It is important to note that this chapter deals only with the major forms of glycosyla-

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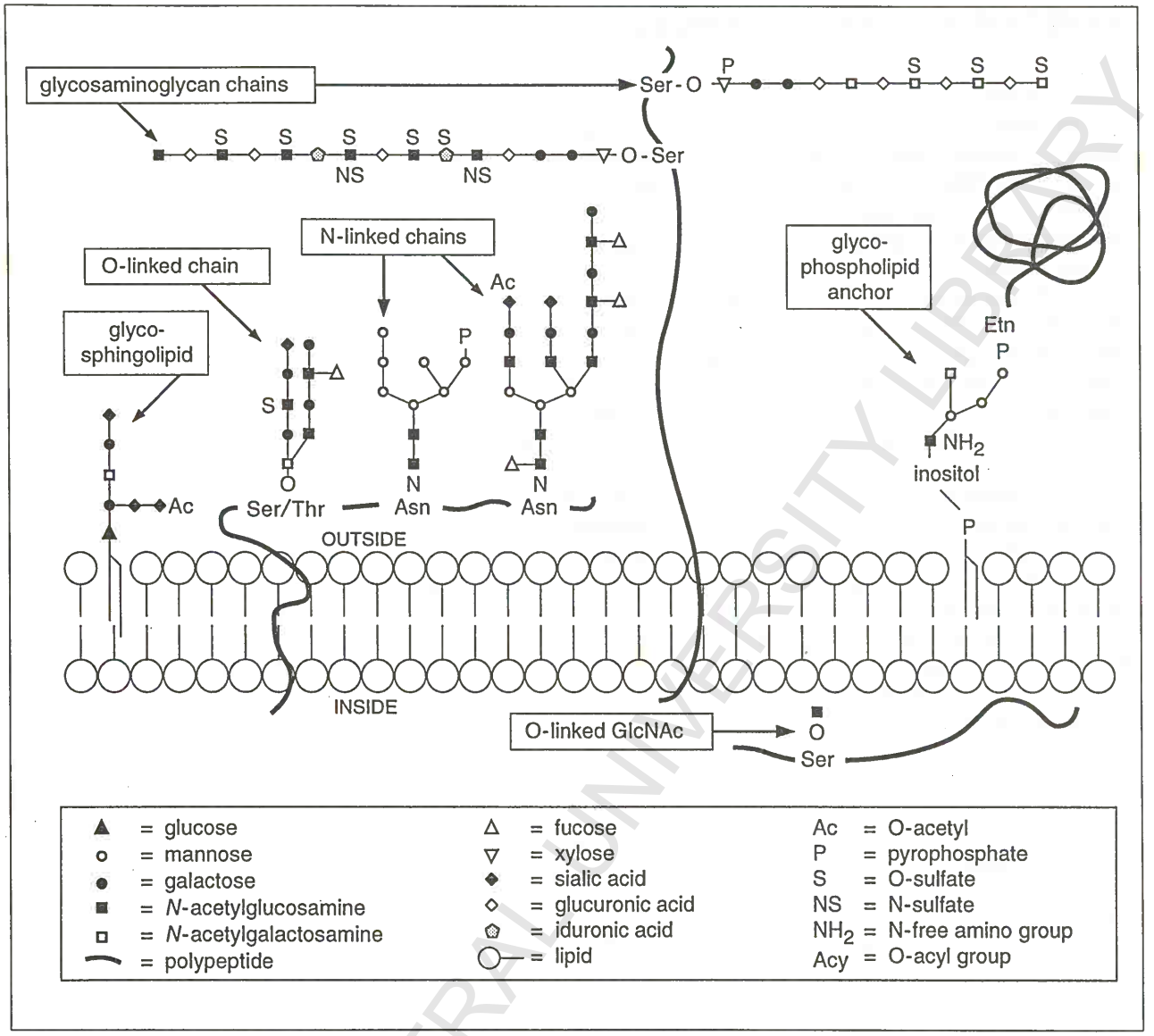

Figure 12.1.1 Common oligosaccharide linkage regions on animal cell glycoconjugates. The most common types of oligosaccharides found in animal glycoconjugates are shown, with an emphasis upon the linkage region between the oligosaccharide and the protein or lipid. Other rarer types of linkage regions and free oligosaccharides that can exist naturally (e.g., hyaluronan) are not shown.

tion found in higher-animal glycoconjugates—*N*-acetylglucosamine (GlcNAc)-*N*-Asn-linked, *N*-acetylgalactosamine (GalNAc)-*O*-Ser/Thr-linked oligosaccharides on glycoproteins, xylose-*O*-Ser-linked glycosaminoglycans on proteoglycans, ceramide-linked glycosphingolipids, phosphatidylinositol-linked glycophospholipid anchors, and O-linked *N*-acetylglucosamine (GlcNAc-*O*-Ser). These structures are depicted in Figure 12.1.1. Other less common forms of glycosylation may need to be considered, especially if prior literature suggests their existence in a given situation. Examples of rarer sugar chains include (1) O-linked glucose, mannose, and fucose; (2) N-linked glucose; (3) glucosyl-hydroxylysine; and (4) GlcNAc-P-Ser (phosphoglycosylation).

Likewise, this chapter deals only with the most common forms of shared outer sequences (e.g., sialylated and fucosylated lactosamines, polylactosamines, *O*-glycosaminoglycan chains, and blood group sequences), and not with rarer sequences (e.g., bisecting xylose residues, N-linked glycosaminoglycans, and β-linked GalNAc residues on N-linked oligosaccharides). For further information, the reader is directed to the Key References section at the end of this unit.

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STEREOCHEMISTRY AND DIAGRAMMATIC REPRESENTATION OF MONOSACCHARIDES AND OLIGOSACCHARIDES

Basic Stereochemistry of Monosaccharides

In addition to this discussion, the reader is referred to Allen and Kisalius (1992) for a more detailed discussion of the principles of carbohydrate structure. The italicized terms in the discussion below are defined in the glossary at the end of this unit.

Glyceraldehyde, the simplest monosaccharide, has a single chiral (asymmetric) carbon (C-2). Therefore, it is a chiral molecule that shows optical isomerism; it can exist in the form of two nonsuperimposable mirror images called *enantiomers* (see Fig. 12.1.2). These enantiomers have identical physical properties, except for the direction of rotation of the plane of polarized light (–, left hand; +, right hand). Historically, the (+)-glyceraldehyde was arbitrarily assigned the prefix D (for dextrorotatory), and the (–)-glyceraldehyde, the prefix L (for levorotatory). The pair of enantiomers also have identical chemical properties, except toward optically active reagents. This fact is particularly important in biological systems, because most enzymes and the compounds they work on are optically active.

The configuration of the highest-numbered asymmetric carbon atom in the carbon chain of a higher monosaccharide is determined by comparison with the configuration of the chiral (or asymmetric) carbon of glyceraldehyde. Thus, a prefix D- is added to the name of each monosaccharide having the configuration of D-glyceraldehyde at the highest-numbered asymmetric carbon, and the prefix L- to those having the configuration of L-glyceraldehyde at the highest-numbered asymmetric carbon. The direction in which the compound rotates the plane of polarized light needs to be determined experimentally, and is indicated by a – or + sign, in parentheses, immediately after the prefix D or L (e.g., D-(+)-glucose). In biological systems, where stereochemical specificity is the rule, D molecules may be completely active and L molecules completely inert, or vice versa. All known naturally occurring monosaccharides in animal cells are in the D configuration, except for fucose and iduronic acid, which are in the L configuration.

Each *aldose* sugar is usually present in a cyclic structure, because the *hemiacetal* produced by reaction of the aldehyde group at C-1 with the hydroxyl group at C-5 gives a six-membered ring, called a *pyranose* (see Fig. 12.1.3). When five-membered rings are formed by reaction of the C-1 aldehyde with the C-4 hydroxyl, they are called *furanoses*. Hexoses commonly form pyranosidic rings, and pentoses form furanosidic rings, although the reverse is possible. Similarly, ketoses (e.g., sialic acids) can form *hemiketals* by reaction of the keto group at C-2 with the hydroxyl group at C-6.

These ring forms are the rule in intact oligosaccharides. The formation of the ring produces an additional chiral center at C-1 (or C-2 for keto sugars). Thus, two additional isomers are possible, which are called *anomers*. These anomers are designated α and β. Because glycosidic linkages (see above) occur via anomeric centers, they are called α linkages and β linkages.

Diagrammatic Representations of Monosaccharides

Monosaccharides are conventionally presented using the Fisher projection or the Haworth representation. Some examples are shown in Figure 12.1.4.

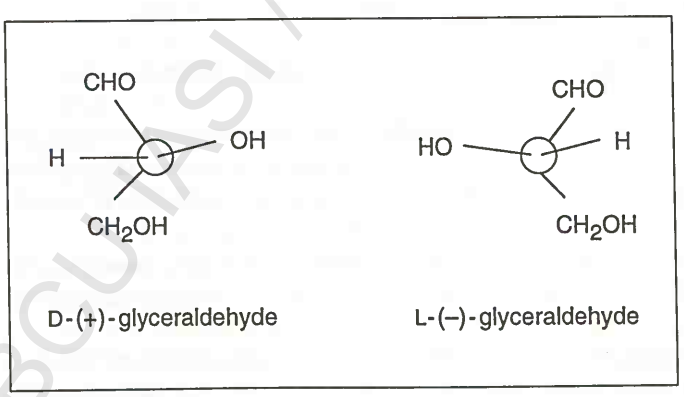

Figure 12.1.2 Enantiomers of glyceraldehyde.

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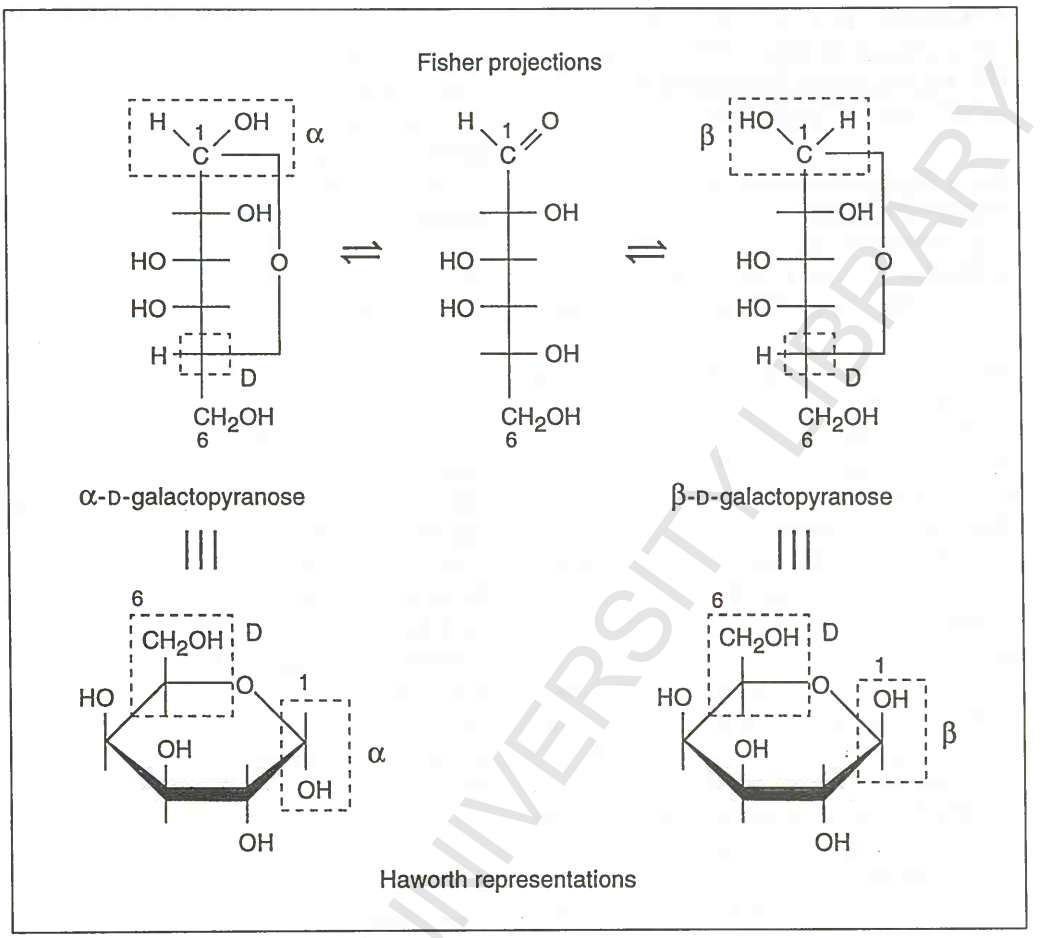

Figure 12.1.3 Formation of cyclic structures: open and ring forms of galactose. The first and last carbon in each chain have been numbered as 1 and 6, respectively. The spatial arrangement of groups that determine the series (D or L) and the anomericity (α and β) of the monosaccharides is indicated by dotted rectangles.

The Fisher projection

The carbon chain of a monosaccharide is written vertically with carbon atom number 1 at the top. The horizontal lines represent bonds projecting out from the plane of the paper, whereas the vertical lines represent bonds projecting behind the plane of the paper. When the hydroxyl group at the highest-numbered asymmetric carbon is on the right, the monosaccharide belongs to the D series. When the hydroxyl group at the highest-numbered asymmetric carbon is on the left, the monosaccharide belongs to the L series.

The Haworth representation

The six-membered ring is represented with the oxygen at the upper right corner, approximately perpendicular to the plane of the paper, and with the groups attached to the carbons above or below the ring. All groups that appear to the right in a Fisher projection appear below the plane of the ring in a Haworth representation. The D-series hexoses have the carboxymethyl group (C-6) above the ring. The reverse is true for the L series. Therefore, the only difference betwen the α and β anomers of a given hexose would be the relative position of the hydroxyl and the hydrogen at C-1. In the α anomer, the hydrogen is below the ring for the D series (above the ring for the L series); in the β anomer the hydrogen is above the ring for the D series (below it for the L series).

Conformation of Monosaccharides in Solution

The Haworth representation does not depict the real conformation of these molecules. The preferred conformation in solution of the six-membered ring monosaccharides is what is known as a chair conformation. In this conformation, each different group can adopt either an *equatorial* or *axial* position.

There are two possible chair conformations called *conformers*, that exist in equilibrium.

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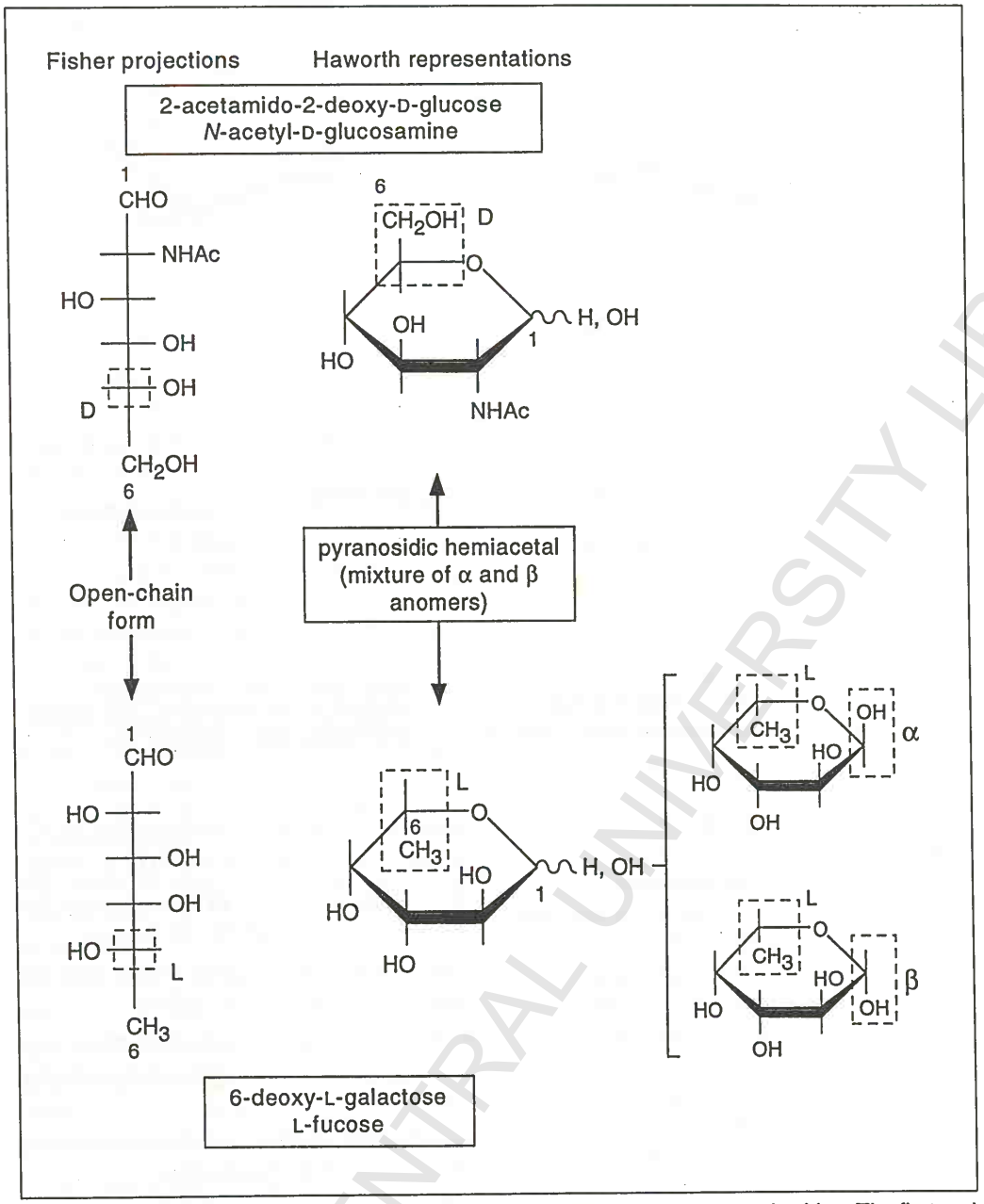

Figure 12.1.4 Fisher projections and Haworth representations of monosaccharides. The first and last carbon in each chain have been numbered as 1 and 6, respectively. The spatial arrangement of groups that determine the series (D or L) of the monosaccharides, and the anomericity (α or β), is indicated with dotted rectangles.

The position of this equilibrium differs from one monosaccharide to another depending on the relative positions of hydroxyl groups or other substituents. The preferred conformation is that with the lowest number of bulky groups in an axial position (Fig. 12.1.5).

Formulas for Representation of Oligosaccharide Chains

Full and correct representation of the formula of an oligosaccharide chain requires notation describing the complete stereochemistry of the component monosaccharides. Thus, the simple tetrasaccharide commonly called sialyl-Lewis^x (attached to an underlying oligosaccharide, R) would be written as follows:

α-D-Neu*p*5Ac-2,3β-D-Gal*p*1,4(α-L-Fuc*p*1,3)-β-D-Glc*p*NAc-R

In more common practice, the D and L configurations and the nature of ring structures are assumed, and the formula is written in one of the ways shown in Figure 12.1.6.

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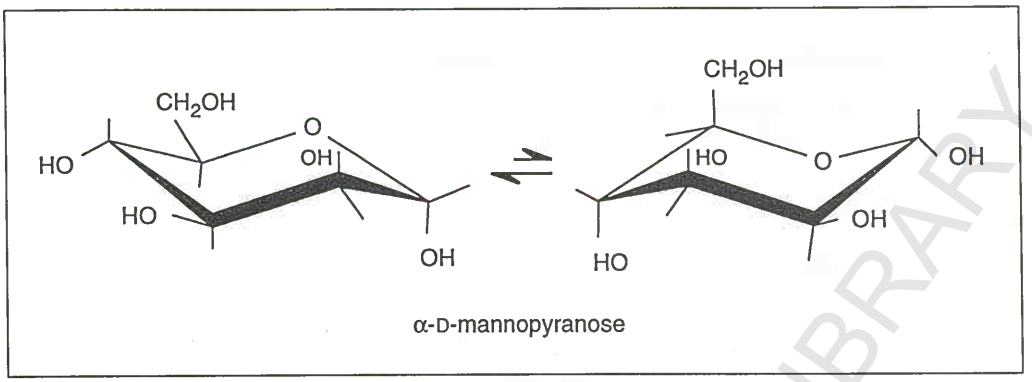

Figure 12.1.5 D-Mannose chair conformation.

Neu5Ac α2,3Galβ1,4(Fuc α1,3)GlcNA c β1–R

Neu5Ac α2–3Gal*p*β1–4GlcNAc β1–R
3
|
1
Fuc α

Figure 12.1.6 Two common short forms for denoting oligosaccharide structures. The tetrasaccharide known as sialyl-Lewis^x, is shown attached to an underlying oligosaccharide, R.

Higher-Order Structures in Oligosaccharides

The presence of monosaccharides with different types of glycosidic linkages in oligo- and polysaccharides creates further complexity, causing the development of secondary and tertiary structures in these molecules. The approximate shape adopted in solution by a given carbohydrate chain can be predicted according to the type of glycosidic linkages involved. These higher-order structures may be critical for the biological roles of glycoconjugates.

GLOSSARY

The following definitions are presented for the terms most commonly used throughout this chapter.

aldose monosaccharide with a carbonyl group at the end of the carbon chain (aldehyde group); the carbonyl group is assigned the lowest possible number (i.e., carbon 1).

carbohydrates polyhydroxyaldehydes or polyhydroxyketones, or compounds that can be hydrolyzed to them.

diastereoisomers compounds with identical formulas that have a different spatial distribution of atoms (e.g., galactose and mannose).

enantiomers nonsuperimposable mirror images of any compound (e.g., D- and L-glucose).

epimers two monosaccharides differing only in the configuration of a single chiral carbon.

Fuc (L-fucose) type of deoxyhexose (see also *types of monosaccharides* below).

Gal (D-galactose) type of hexose (see also *types of monosaccharides* below).

GalNAc (N-acetyl-D-galactosamine) type of hexosamine (see also *types of monosaccharides* below).

ganglioside anionic glycolipid containing one or more units of sialic acid.

Glc (D-glucose) type of hexose (see also *types of monosaccharides* below).

GlcNAc (N-acetyl-D-glucosamine) type of hexosamine (see also *types of monosaccharides* below).

glyceraldehyde simplest monosaccharide (an aldotriose; i.e., containing three carbon atoms).

glycoconjugate natural compound in which one or more monosaccharide or oligosaccharide units are covalently linked to a noncarbohydrate moiety.

GlUA or GlA (D-glucuronic acid) type of uronic acid (see also *types of monosaccharides* below).

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glycolipid or glycosphingolipid oligosaccharide attached via glucose or galactose to the terminal primary hydroxyl group of ceramide, which is composed of a long-chain base (i.e., sphingosine) and a fatty acid. Glycolipids can be neutral or anionic (negatively charged).

glycophospholipid anchor glycan bridge between phosphatidylinositol and a phosphoethanolamine in amide linkage to the C terminus of a protein; constitutes the sole membrane anchor for such proteins.

glycoprotein glycoconjugate in which a protein carries one or more oligosaccharide chains covalently attached to a polypeptide backbone via *N*-GlcNAc- or *O*-GalNAc-linkages.

glycosaminoglycan linear copolymers of disaccharide repeating units, each composed of a hexosamine and a hexose or hexuronic acid; these are the oligosaccharide chains that define the proteoglycans. The type of disaccharide unit can define the glycosaminoglycans as chondroitin or dermatan sulfate, heparan or heparin sulfate, hyaluronic acid, and keratan sulfate. The glycosaminoglycans (except hyaluronic acid) also contain sulfate esters substituting either hydroxyl or amino groups (*N*- or *O*-sulfate groups).

glycosidic linkage linkage of a monosaccharide to another residue via the anomeric hydroxyl group.

IdUA or IdA (L-iduronic acid) type of uronic acid (see also *types of monosaccharides* below).

ketose monosaccharide with a carbonyl group in an inner carbon; the carbonyl group is assigned the lowest possible number (i.e., carbon 2).

Man (D-mannose) type of hexose (see also *types of monosaccharides* below).

monosaccharide carbohydrate that cannot be hydrolyzed into simpler units (see also *types of monosaccharides* below).

mucin large glycoproteins that contain many (up to several hundred) *O*-GalNAc-linked oligosaccharide chains that are often closely spaced.

N-linked oligosaccharide oligosaccharide covalently linked to an asparagine residue of a polypeptide chain in the consensus sequence -Asn-X-Ser/Thr. Although there are many different kinds of N-linked oligosaccharides, they have certain common features: (1) a common core pentasaccharide containing two mannosyl residues α-linked to a third mannosyl unit, which is in turn β-linked to a chitobiosyl group and (2) a chitobiosyl group that is β-linked to the asparagine amide nitrogen. The N-linked oligosaccharides can be divided into three main classes: high-mannose type, complex type, and hybrid type.

Neu5Ac (N-acetyl-D-neuraminic acid) type of sialic acid (Sia; see also *types of monosaccharides* below).

O-linked oligosaccharide oligosaccharide linked to the polypeptide via *N*-acetylgalactosamine (GalNAc) to serine or threonine. Note that other types of O-linked oligosaccharides also exist (e.g., *O*-GlcNAc) but that the *O*-GalNAc linkage, being the most well-known, is often described by this generic term.

oligosaccharide branched or linear chain of monosaccharides attached to one another via glycosidic linkages (see also *polysaccharides* below).

polylactosaminoglycan long chain of repeating units of the disaccharide β-Gal(1-4)-GlcNAc. These chains may be modified by sialylation, fucosylation, or branching. When sulfated, they are called keratan sulfate (see *glycosaminoglycans* above).

polysaccharides branched or linear chain of monosaccharides attached to each other via glycosidic linkages that usually contain repetitive sequences. The number of monosaccharide residues that represents the limit between an oligosaccharide and a polysaccharide is not defined. A tetrafucosylated, sialylated tetraantennary carbohydrate moiety containing eighteen monosaccharide units, present in a glycoprotein, is considered an oligosaccharide. On the other hand, a carbohydrate composed of eighteen glucose residues linked β-(1-4) present in a plant extract is considered a polysaccharide.

polysialic acid homopolymer of sialic acid selectively expressed on a few vertebrate proteins and on the capsular polysaccharides of certain pathogenic bacteria.

proteoglycan glycoconjugate having one or more *O*-xylose-linked glycosaminoglycan chains (rather than *N*-GlcNAc- or *O*-GalNAc-linked oligosaccharides) linked to protein. The distinction from a glycoprotein is otherwise arbitrary, because some proteoglycans can have both glycosaminoglycan chains and N- or O-linked oligosaccharides attached to them.

reducing sugar sugar that undergoes typical reactions of aldehydes (e.g., is able to reduce

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Ag^+ or Cu^{++}). Mono-, oligo-, or polysaccharides can be reducing sugars when the aldehyde group in the terminal monosaccharide residue is not involved in a glycosidic linkage.

saccharide modifications hydroxyl groups of different monosaccharides can be subject to phosphorylation, acetylation, sulfation, methylation, or fatty acylation. Amino groups can be free, *N*-acetylated, or *N*-sulfated. Carboxyl groups are occasionally subject to lactonization to nearby hydroxyl groups.

Sia (sialic acid) generic name for a family of acidic nine-carbon monosaccharides (e.g., Neu5Ac).

types of monosaccharides monosaccharides may have a carbonyl group at the end of the carbon chain (aldehyde group) or in an inner carbon (ketone group). The carbonyl group is assigned the lowest possible number, e.g., carbon 1 (C-1) for the aldehyde group; carbon 2 (C-2) for the most common ketone groups. These two types are named aldoses and ketoses accordingly. The simplest monosaccharide is glyceraldehyde (see Fig. 12.1.2), an aldotriose (i.e., containing three carbon atoms). Natural aldoses with different number of carbon atoms in their chain are named accordingly (e.g., aldohexoses, containing six carbon atoms). Two monosaccharides differing only in the configuration of a single chiral carbon are called epimers. For example, glucose and galactose are epimers of each other at C-4. The common monosaccharides present in animal glycoconjugates are (1) deoxyhexoses (e.g., L-Fuc); (2) hexosamines, usually N-acetylated (e.g., D-GalNAc and D-GlcNAc); (3) hexoses (e.g., D-Glc, D-Gal, and D-Man); (4) pentoses (e.g., D-Xyl); (5) sialic acids (Sia; e.g., Neu5Ac); and (6) uronic acids (e.g., D-GlA and L-IdA).

Xyl (D-xylose) type of pentose (see also *types of monosaccharides* above).

For further detailed information on the analysis of oligosaccharides, the reader is referred to the specific literature cited in the individual protocol units. In addition, the following sources can be used for general information, for details on specific methods, and for many additional methods that are not included in this chapter.

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Metabolic Radiolabeling of Animal Cell Glycoconjugates

Useful information about glycoconjugates can be obtained by labeling their aglycone (noncarbohydrate) portions—e.g., labeling proteins with radioactive amino acids—then using techniques described elsewhere in this chapter to infer the presence, type, and nature of oligosaccharide chains. This unit describes metabolic labeling techniques that provide more specific information about the structure, sequence, and distribution of the sugar chains of glycoconjugates. Following metabolic labeling, the radioactive glycoconjugate of interest is isolated, individual glycosylation sites are identified and separated if necessary, and the labeled oligosaccharides are subjected to structural analysis. Although these techniques provide less information than would complete sequencing of the sugar chains, the partial structural information derived is sufficient for many purposes.

Metabolic labeling is simple and easy to perform, and requires no sophisticated instrumentation other than a scintillation counter. In addition, purification of the glycoconjugate to radiometric homogeneity is sufficient for further analysis (i.e., other contaminating molecules that are not labeled do not have to be removed). Important practical considerations for metabolic labeling experiments include selecting the type of experiment and the labeled precursor, understanding the specificity of labeling, and maximizing uptake and incorporation (see Critical Parameters). Before proceeding with experiments to label glycoconjugates metabolically, labeling conditions should be evaluated to ensure that cell viability and metabolism are not altered, and optimized for uptake and incorporation of the precursor (this should be done for each combination of precursor and cell line; see Basic Protocol and Alternate Protocol 1).

In the Basic Protocol, cells in culture are grown through several population doublings in complete medium supplemented with radiolabeled glycoconjugate precursors to reach a steady-state level of incorporation. In the alternate protocols, cells are cultured for a short period of time in a deficient medium that contains a high concentration of radiolabeled precursor. A pulse or pulse-chase labeling procedure (Alternate Protocol 1) can be used to analyze precursor-product relationships. With sequential pulse-labeling (Alternate Protocol 2), it is possible to obtain quantities of labeled glycoconjugates while using a minimum amount of labeled precursor by using the same batch of medium to pulse-label a series of cultures. A support protocol describes the preparation of multiply deficient medium (MDM) for use in making appropriate deficient media.

NOTE: Conventional protocols for handling, monitoring, shielding, and disposing of radioactivity and radioactive waste (*APPENDIX 2B*) and for tissue culture of cells and sterile handling of media should be followed throughout these protocols.

STEADY-STATE LABELING WITH RADIOACTIVE PRECURSORS

BASIC PROTOCOL

When studying glycoconjugates in an established tissue culture cell line, molecules of interest can be labeled for structural characterization. In the following protocol, cells in culture are grown to a steady-state level of incorporation in complete medium supplemented with radiolabeled glycoconjugate precursor.

Optimizing Conditions

An attempt should be made to label the molecules as close as possible to the metabolic steady state—i.e., a constant level of radioactivity per mass unit of a given monosaccharide in all glycoconjugates in the cell—under conditions of normal growth. In practice, this is somewhat difficult to achieve with most cell types. The maximum possible number

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of cells should be grown for the longest possible period of time in the minimum possible volume of complete medium supplemented with the maximum possible amount of radioactive label. Before labeling is attempted, the following growth characteristics should be determined for the cell type of interest: (1) population doubling time, (2) maximum degree of dilution upon splitting that is compatible with proper regrowth, (3) maximum cell density compatible with healthy growth and metabolism (confluence), and (4) minimum volume of medium that will sustain regrowth from a full split to confluence. If these parameters are properly defined, growth from full split to confluence will allow at least three population doublings during the labeling period for most cell lines. In some cases, however (e.g., with very slowly growing cell lines, lines requiring frequent medium changes, or cells that cannot be diluted to low density when splitting), this may not be feasible.

Materials

Radioactive precursor: ^{3}H- or ^{14}C-labeled monosaccharide, [^{35}S]sulfate, [^{3}H]acetate, or [^{32}P]orthophosphate; at highest available specific activity
Complete tissue culture medium appropriate for long-term growth of tissue culture cell line, supplemented as necessary
Established tissue culture cell line, either suspension or monolayer
PBS, pH 7.2, (*APPENDIX 2E*), ice cold

Disposable 50-ml vacuum-suction filter device: filter flask fitted with 0.22-μm filter
Disposable 0.22-μm filter attached to sterile plastic syringe, both with Luer-Lok fittings
Tissue culture plates or flasks
Screw-cap centrifuge tubes
Tabletop centrifuges, at room temperature and 4°C

1. If necessary, dry radioactive precursor (in a ventilated hood) to completely remove any organic solvents. Dissolve in a small volume of complete tissue culture medium.

2. Filter sterilize radioactive medium, using a standard disposable 50-ml vacuum suction filter device (filter flask fitted with a 0.22-μm filter) for volumes ≥10 ml or a 0.22-μm filter attached directly to the tip of a disposable sterile syringe for smaller volumes. Wash out the original container with additional medium and use this fluid to wash through the filter (this maximizes recovery of label).

   CAUTION: *Dispose of the radioactive filter and/or syringe appropriately.*

   The radioactive precursor may be available in a sterile aqueous medium, ready for use. However, such preparations are more expensive, and after repeated opening of such packages, filtering is recommended to ensure continued sterility.

3. Add additional medium if necessary to bring the volume to the amount needed for the experiment. Warm the medium in an incubator or water bath to the temperature at which labeling will be done.

4. Using a sterile pipet tip, aliquot a small amount of radioactive medium and save for scintillation counting in step 8.

5. Split monolayer cells according to standard method (e.g., detach by trypsinization), resuspend in radioactive medium, and plate onto tissue culture plates. For suspension cultures, dilute directly in radioactive medium or concentrate as follows: place culture in an appropriately sized screw-cap centrifuge tube and centrifuge in a tabletop centrifuge 5 min at ~500 × *g*, room temperature. Discard supernatant and resuspend

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cell pellet in radioactive medium. Incubate under standard conditions for the cell line being studied.

The number of cells needed, and therefore the culture vessels and volume of medium used, will depend on the growth characteristics of the particular cell line being studied and should be optimized as described in the protocol introduction.

6. When the cells reach maximum growth (confluence or late-log phase), chill culture on ice and harvest cells, scraping monolayer cells from plate using a rubber policeman or cell scraper. Pellet cells 5 min at ~500 × *g*, 4°C. Decant the labeled medium and save an aliquot for scintillation counting in step 8.

   If the glycoconjugates in the conditioned medium are to be studied, the medium should be filtered through a 0.22-μm filter to remove any cell debris remaining after the centrifugation.

7. Wash cell pellet twice in a >50-fold excess of ice-cold PBS, pH 7.2.

   At this stage, the labeled cell pellet can be frozen at –20°C to –80°C for later analysis if desired.

8. Using a scintillation counter, determine the efficiency of incorporation by measuring the radioactivity incorporated into an aliquot of the cells and the sample of conditioned medium from step 6. As a control, measure the radioactivity of the sample of sterile radioactive medium from step 4.

   It may be necessary to dilute the medium to get an accurate count.

PULSE OR PULSE-CHASE LABELING WITH RADIOACTIVE PRECURSORS

ALTERNATE PROTOCOL 1

If steady-state labeling (see Basic Protocol) does not yield sufficient incorporation of label into the glycoconjugate of interest, it may be desirable to provide labeled precursor in the absence of, or with decreased amounts of, the unlabeled form. The pulse procedure described in this protocol—in which cells are cultured briefly in deficient medium supplemented with a radioactive precursor—may be used in these cases. Alternatively, the procedure can be expanded into a pulse-chase by adding a chase of nonradioactive medium; this permits brief labeling of the glycoconjugate of interest to establish precursor-product relationships. These procedures are most useful for labeling monosaccharides that compete with glucose for uptake—i.e., Gal, Glc, Man, and $GlcNH_2$ (see Commentary). They are of limited value for monosaccharides that are not usually taken up efficiently by cells—i.e., GlcNAc, GalNAc, ManNac, Neu5Ac, Fuc, Xyl, and GlcA—unless very large quantities of such labeled molecules can be used for pulse labeling. Because pulse labelings are typically done for a short time (e.g., minutes or hours), cells are usually used in a nearly confluent state, to maximize uptake and incorporation.

Optimizing Conditions

Before the experiment is performed, several pilot experiments should be carried out to determine the optimal conditions for labeling.

Determining base incorporation level and the effect of glucose. MDM (multiply deficient medium) appropriate for growing cultures of the cell line of interest should be reconstituted (see Support Protocol) to 100% levels of all components except the one being presented as a radiolabeled precursor or competing molecule (e.g., glucose). If required by the cells, dialyzed serum should be added to the appropriate final concentration. Finally, radiolabeled precursor should be added. A small-scale pilot labeling should be carried out by culturing cells in the labeled medium and monitoring incorporation of the label into the macromolecule of interest or into whole-cell glycoproteins. If the

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radiolabeled precursor is a monosaccharide, the effect of glucose on incorporation of radiolabeled precursor should be assessed by carrying out this pilot experiment in duplicate, using one lot of medium prepared as described and one prepared the same way but without glucose.

Determining optimal concentration of radiolabeled precursor. Enough MDM for several pilot experiments should be reconstituted to 100% levels of all components except the one being presented as a radiolabeled precursor. The radiolabeled precursor should then be added and the medium divided into aliquots. The aliquots should be supplemented with an unlabeled 100× stock solution of the same precursor to yield a series of media containing different concentrations of the unlabeled precursor (e.g., 0%, 5%, 10%, 20%, 50%, and 100% of the concentration in normal medium). These should be used for small-scale pilot labelings of the cell line of interest for defined periods of time and incorporation of label should be monitored as described above. Incorporation should be plotted against the total precursor concentration. The point at which the curve breaks—i.e., where the percentage of label incorporated is markedly decreased by further addition of unlabeled compound—is the point at which the precursor concentration is no longer limiting for biosynthetic reactions (Fig. 12.2.1). The optimal concentration of unlabeled precursor will be a little higher than the break point for the curve, where unlabeled precursor is not limiting but incorporation is still good.

Checking cell growth at optimal precursor concentration. Reconstituted MDM containing radioactive precursor plus the optimal concentration of unlabeled precursor should be prepared and cells should be grown in this medium for the desired period of time. Incorporation of radiolabeled precursor should be monitored and linearity of uptake and incorporation of label over time should be assessed. Measures of cell growth, stability, and general biosynthetic capability—e.g., cell counts and trypan blue exclusion (*UNIT 5.5*) and radioactive amino acid incorporation—should also be assayed to determine if reducing the concentration of the precursor into this range has any detrimental effect upon the

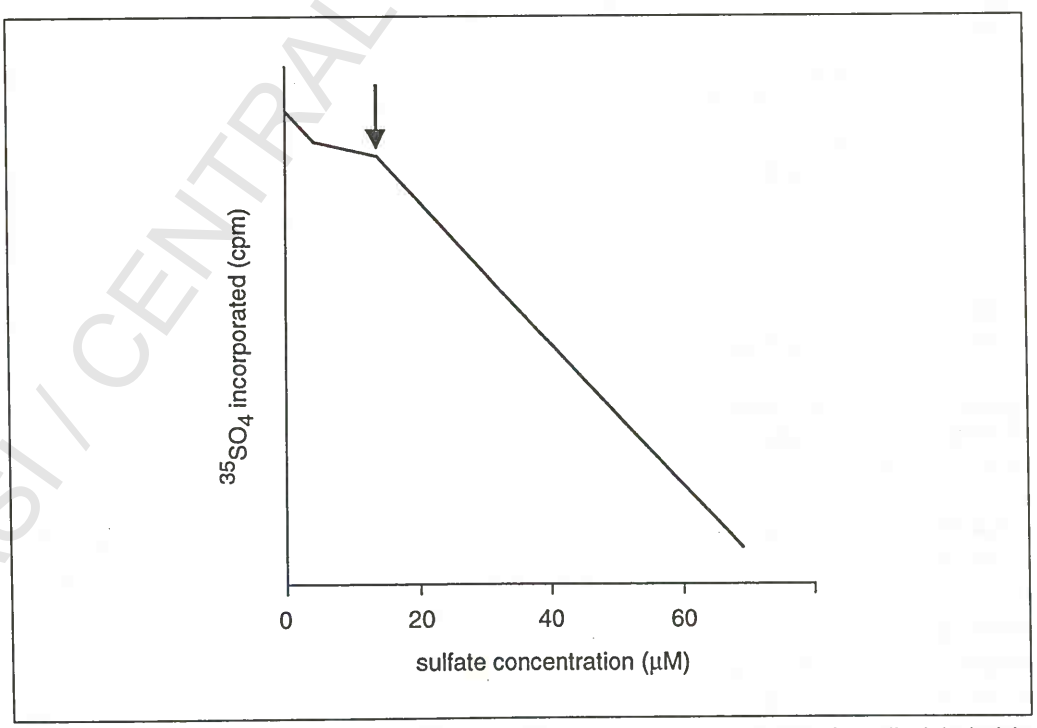

Figure 12.2.1 Incorporation of radiolabeled sulfates into macromolecules in cells labeled in sulfate-free medium and the effect of adding increasing amounts of unlabeled sulfate. The arrow indicates the point at which the sulfate is no longer limiting.

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cells. It is sometimes necessary to reach a compromise between the opposing factors of lowered concentration, labeling time, and cell viability. An attempt should be made to determine the lowest concentration of the unlabeled precursor that can be used for the desired period of time without affecting cell growth, viability, and biosynthesis of macromolecules in general. It should be kept in mind that the use of partially deficient medium may selectively alter glycoconjugate makeup (e.g., lowering sulfate concentration too much can result in undersulfation of glycosaminoglycans in some cell types).

Additional Materials (also see Basic Protocol)

Multiply deficient medium (MDM; see Support Protocol and Table 12.2.1), supplemented as appropriate

Fetal bovine serum (FBS; GIBCO/BRL), dialyzed (*APPENDIX 3B*) against sterile 0.15 M NaCl (glucose concentration ~250 μM)

Table 12.2.1 Composition of Multiply Deficient Medium (MDM)

Component	Final concentration in complete medium	Stock[a]
$CaCl_2$	200 mg/l	Salt, reagent grade
KCl	400 mg/l	Salt, reagent grade
$MgCl_2$[b]	75 mg/l	Salt, reagent grade
NaCl	6800 mg/l	Salt, reagent grade
$NaH_2PO_4 \cdot H_2O$	140 mg/l	Salt, reagent grade
Phenol red	10 mg/l	10×
Sodium pyruvate	110 mg/l	100×
L-Alanine	25 mg/l	100×
L-Arginine	126 mg/l	100×
L-Asparagine	50 mg/l	100×
L-Aspartic acid	30 mg/l	100×
L-Cysteine	NONE[c]	—
L-Glutamic acid	75 mg/l	100×
L-Glutamine	NONE[c]	—
L-Glycine	50 mg/l	100×
L-Histidine	42 mg/l	100×
L-Isoleucine	52 mg/l	100×
L-Leucine	52 mg/l	100×
L-Lysine	72 mg/l	100×
L-Methionine	NONE[c]	—
L-Phenylalanine	32 mg/l	100×
L-Proline	40 mg/l	100×
L-Serine	NONE[c]	—
L-Threonine	48 mg/l	100×
L-Tryptophan	10 mg/l	100×
L-Tyrosine	36 mg/l	100×
L-Valine	46 mg/l	100×
MEM vitamins (mixture)	1×	100×

[a]10× and 100× stock solutions are available commercially (e.g., from GIBCO/BRL).

[b]Do not use $MgSO_4$ in place of $MgCl_2$.

[c]Not present in MDM; add as needed for specific experiments.

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1. Determine optimal conditions for labeling as described in the protocol introduction. Grow monolayer cells in complete medium to near confluence or suspension cells to late-log phase.
2. Based on the pilot experiments for optimizing conditions described in the protocol introduction, reconstitute MDM to prepare a stock of medium partially deficient in only the precursor of interest.
3. Add an appropriate quantity of radioactive precursor to the reconstituted MDM. Add dialyzed FBS if required for growth of the cell line being studied. Filter sterilize, warm the medium to 37°C, and reserve an aliquot of radioactive medium (see Basic Protocol, steps 2 to 4).
4. For monolayer cells, aspirate culture medium from plate; for suspension cells, microcentrifuge or centrifuge briefly and decant the supernatant medium. Add an appropriate quantity of radioactive labeling medium from step 3. Incubate cells for the desired period of time.

   Labeling time should be based on the length of time the cells can survive in deficient medium as determined in the optimizing pilot experiment.

   Save radioactive medium for analysis, if necessary.
5. For pulse labeling, proceed to step 6. For pulse-chase labeling, remove medium as described in step 4, add a chase of nonradioactive complete medium, and incubate for the desired period of time.

   For a pulse-chase experiment, several vessels of each cell culture should be set up and chased for varying periods of time.
6. Harvest and wash the cells. Determine efficiency of incorporation (see Basic Protocol, steps 6 to 8).

ALTERNATE PROTOCOL 2

SEQUENTIAL PULSE OR PULSE-CHASE LABELING WITH REUSE OF RADIOACTIVELY LABELED MEDIUM

In some cases, incorporation of label into the glycoconjugate of interest may be inadequate, even under defined conditions with selectively deficient medium. In addition, prolonged exposure to deficient medium may result in alterations in synthesis of proteins or other macromolecules. In these situations, it may be desirable to expose a series of plates of cells sequentially to a small volume of medium containing a high concentration of label. This also conserves expensive radiolabeled precursor. In this protocol, multiple sets of cell cultures are sequentially pulse-labeled. One set is incubated in labeling medium for the pulse period, then the labeling medium is removed and added to the next set. For a pulse experiment (designed to obtain a sizable quantity of material with a high level of incorporation), the cells are harvested at this point; for a pulse-chase, a chase of nonradioactive complete medium is added to the first set of cultures. This process is repeated until all cells have been labeled (Fig. 12.2.2). Because the incubation period is relatively short (e.g., a few hours), it is assumed that only a very small fraction of the radiolabeled precursor is consumed from the medium during each labeling cycle and that the concentrations of other essential components are not substantially changed. To further conserve labeled medium, some investigators freeze it after limited usage and thaw it to reuse (the pH of thawed medium should be adjusted before use). When reusing medium, experimental results should be interpreted carefully, especially with regard to the specific activity of labeled products and the possibility of metabolic effects of secreted molecules.

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Additional Materials (also see Basic Protocol)

Multiply deficient medium (MDM; see Support Protocol and Table 12.2.1), supplemented as appropriate

Fetal bovine serum (FBS; GIBCO/BRL), dialyzed (*APPENDIX 3B*) against sterile 0.15 M NaCl (glucose concentration ~250 µM)

1. Determine optimal conditions for labeling (see Alternate Protocol 1 introduction). Set up several identical sets of monolayer or suspension cells to be labeled sequentially and grow to confluence or late-log phase.

   For suspension cultures in which it is possible to use <1 ml labeling medium, cells may be labeled in tightly capped 1.5-ml screw-cap microcentrifuge tubes and incubated at 37°C on a rotating end-over-end mixer.

2. Prepare radioactive labeling medium and label the first set of cell cultures for the appropriate length of time (see Alternate Protocol 1, steps 2 to 4).

   Labeling time will vary from minutes to hours, depending upon the cell type and the objective of the labeling.

3. For monolayer cells, aspirate the radioactive medium from the first set of cultures; for suspension cells, microcentrifuge briefly (~10 sec at top speed) and decant the supernatant radioactive medium. Transfer the radioactive medium to the second set of cultures.

4. For pulse labeling, harvest cells from first set of cultures. For pulse-chase labeling, add a chase consisting of an adequate quantity of nonradioactive complete culture medium to the first set of cultures and continue incubating.

5. Repeat transfers until all sets of cells have been labeled.

6. Harvest all plates. For pulse labeling, pool all the pellets together. For pulse-chase labeling, keep the individual cell pellets separate (see Fig. 12.2.2).

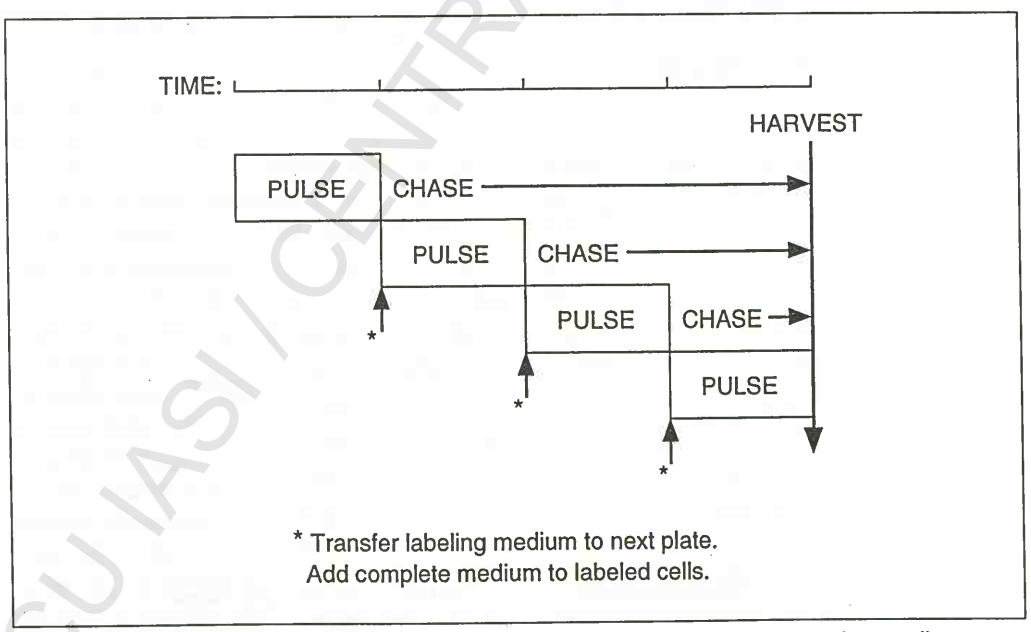

Figure 12.2.2 Scheme for sequential pulse labeling of cells reutilizing radioactive medium.

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SUPPORT PROTOCOL

PREPARATION AND SUPPLEMENTATION OF MULTIPLY DEFICIENT MEDIUM (MDM)

Some types of selectively deficient media are available commercially at reasonable prices, and some companies will custom-prepare selectively deficient media on request. For a laboratory where labeling experiments with a variety of deficient media are frequently carried out, it is convenient to prepare a basic multiply deficient medium (MDM) that is completely lacking in several commonly studied components. This medium can be used to make up different selectively deficient media as needed. The following MDM, based on α-MEM medium (GIBCO/BRL), supports growth of most tissue culture cells.

For labeling experiments, MDM can be reconstituted with ^{3}H- or ^{14}C-labeled monosaccharides, [^{35}S]sulfate, ^{35}S-labeled methionine or cysteine, or [^{3}H]serine. If desired, other specific components can also be omitted and replaced with their radiolabeled forms.

NOTE: Tissue culture grade reagents (including distilled, deionized water) should be used in making MDM. To ensure that reagents do not become contaminated by compounds such as endotoxin as portions are being removed from stock bottles, it is important to pour liquids carefully and use disposable spatulas for removing solids. For the same reason, clean glassware must be used; preferably, a set of glassware should be set aside for this purpose. 100× stock solutions of many components can be purchased commercially in sterile form, or may be made up from solids and filter sterilized individually.

Additional Materials (also see Basic Protocol)

Stock solutions for multiply deficient medium (MDM; Table 12.2.1)
100× stock solutions for reconstituting MDM (Table 12.2.2)

1. Based on the experiment to be performed, determine appropriate components for MDM.
2. Make up MDM according to the ingredient list in Table 12.2.1 by dissolving salts and phenol red one by one in ~800 ml water, adding sufficient pyruvate, amino acid, and vitamin stocks for a 1× final concentration in 1 liter, and adding water to 1 liter.
3. Filter sterilize. Do not adjust the pH. Freeze 25-ml aliquots in 50-ml tubes at –20°C.

   The solution will be yellow.

Table 12.2.2 Stock Solutions for Reconstitution of MDM[a]

Component	Final concentration in complete medium	Stock[b]
$NaHCO_3$[c]	1× (2200 mg/l)	100×
HEPES·HCl[c]	20 mM (4.76 g/l)	2 M, pH 7.3
Na_2SO_4	0.81 mM (115 mg/l)	100 mM, sterile
D-Glucose	1× (1000 mg/ml)	100×
L-Cysteine	1× (100 mg/l)	100×
L-Glutamine	1× (292 mg/l)	100×
L-Methionine	1× (15 mg/l)	100×
L-Serine	1× (25 mg/l)	100×

[a]Individual components are added to MDM at full strength, lower concentration, or left out altogether, depending upon the experiment planned.

[b]100× stock solutions are available commercially (e.g., from GIBCO/BRL).

[c]Use either $NaHCO_3/CO_2$ *or* HEPES·HCl (not both); these control the pH of the final medium.

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4. Add appropriate components (from those listed in Table 12.2.2) to reconstitute MDM for the labeling experiment.

The following example describes the selective reconstitution of MDM with missing components. It is a recipe for 50 ml of a supplemented MDM for optimal labeling of endothelial cells with [^{35}S]sulfate and [6-^{3}H]glucosamine, which requires a medium with no cysteine, low glucose, and low concentrations of methionine and sulfate (see Roux et al., 1988, for detailed rationale). Stock solutions are as listed in Table 12.2.2.

To 50 ml MDM, add:

500 µl 100× $NaHCO_3$ stock solution
500 µl 100× glutamine stock solution
500 µl 100× serine stock solution
50 µl 100× glucose stock solution (200 mg/L final)
10 µl 100 mM inorganic sulfate stock solution (20 µM final)
50 µl 100× methionine stock solution (0.1× final).

Just before use, add 0.5 mCi each of [^{35}S]sulfate and [6-^{3}H]glucosamine and mix. Filter sterilize directly into culture vessel. The total volume of reconstituted medium will be slightly more than 50 ml; for practical purposes, this minor discrepancy can be ignored. The medium should be orange-red, indicating the correct pH range.

COMMENTARY

Background Information

If sufficient quantities of pure molecules (i.e., in the nanomole range) are available, complete sequencing of oligosaccharides is best performed by conventional techniques not requiring radioactivity. However, isolation of sufficient quantities of material may not be practical (e.g., in analysis of biosynthetic intermediates or rare molecules). Alternatively, the biological questions underlying the experiment may be adequately answered by less definitive means of structural analysis. In these cases, metabolic labeling with radioactive sugars or donors that can transfer label to sugars may provide sufficient structural information about the oligosaccharide chains (see Cummings et al., 1989).

Sugar chains of glycoconjugates can be successfully labeled by short-term labeling experiments using radioactive precursors (Tabas and Kornfeld, 1980; Goldberg and Kornfeld, 1981; Roux et al., 1988; Muchmore et al., 1989). Although sugar nucleotides are the immediate donors for glycosylation reactions, they cannot be taken up by cultured cells. Hence, metabolic labeling of sugar chains is accomplished by providing the cells with radiolabeled monosaccharides (Yurchenco et al., 1978; Yanagishita et al., 1989; Varki, 1991). These are taken up by the cells, activated to sugar nucleotides, and transported into the Golgi apparatus, where lumenally oriented transferases add the monosaccharides to lumenally oriented acceptors (Hirschberg and Snider, 1987). The few known exceptions to this topology are cytosolic and nuclear forms of glycosylation (Hart et al., 1989). For oligosaccharides that contain modifications—such as sulfate, acetate, and phosphate ester groups—an alternative metabolic labeling technique is to use a [^{35}S]sulfate, [^{3}H]acetate, or [^{32}P]orthophosphate label, respectively.

Although metabolic labeling can provide useful information regarding glycoprotein oligosaccharides, there are significant limitations to its use. First, it is difficult to determine when true steady-state labeling of a cell is reached (as a rule of thumb, 3 to 4 doublings are usually assumed to be sufficient); thus the numerical ratio between labeled glycoconjugates may not reflect steady-state ratios. Second, individual precursors show greatly differing uptake and incorporation in different cell types. Third, almost all labeled precursors are only partially specific for certain monosaccharides, and the degree of this specificity can vary depending on cell type. Fourth, although lowering glucose concentration improves the incorporation of monosaccharides that compete with glucose for uptake, the low glucose supply may also directly affect oligosaccharide precursors in some cell types.

Critical Parameters

As diagrammed in Figure 12.2.3, the labeling protocol most suitable for a particular study depends on several different considerations. Some of these arise from the objectives of the

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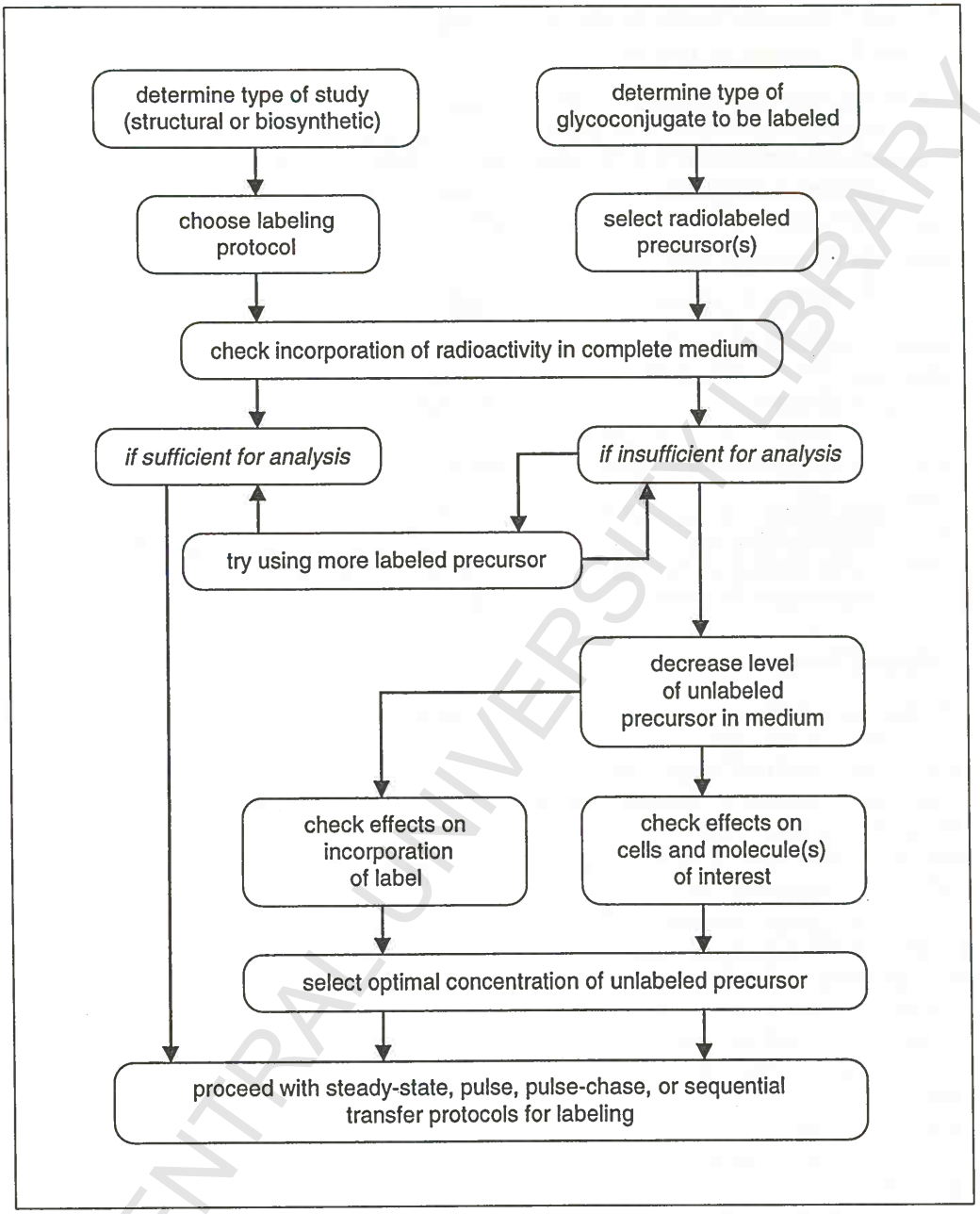

Figure 12.2.3 Strategy for planning metabolic labeling of animal cell glycoconjugates.

study and must be investigated by performing pilot experiments to determine optimal conditions (see Optimizing Conditions sections of the Basic Protocol and Alternate Protocol 1).

General considerations

If the goal of a study is to obtain labeled material for structural identification and partial characterization, the yield of radioactivity should be maximized by either steady-state or pulse labeling. For quantitating the relative mass of a molecule in two different samples or comparing the masses of two molecules in the same sample, the oligosaccharide should be labeled to constant specific activity—i.e., steady-state distribution (although this may not always be achievable). For establishing precursor-product relationships between molecules, a pulse-chase protocol should be used. If the radioactive precursor is expensive, a sequential procedure may be employed in pulse and pulse-chase experiments to minimize the quantity needed.

Selecting a labeled precursor

Selection of the labeled precursor to be used is based upon several factors, including efficiency of uptake (see section on Factors affect-

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Table 12.2.3 Distribution of Monosaccharides and Modifications in Glycoconjugates[a]

Precursor	Type of glycoconjugate					
	N-GlcNAc-linked glycoprotein	*O*-GalNAc-linked glycoprotein	Xylose-linked proteoglycan	Glyco-sphingolipid	Glyco-phospholipid anchor	O-linked GlcNAc
Man	+++	–	–	–	+++	–
Fuc	++	++	+/–	+	–	–
Gal	+++	+++	++	+++	+/– (side chain)	–
Glc	+ (precursor)	–	–	++	–	–
GlcNAc	+++	+++	++	++	+ (free amine)	++
GalNAc	+/–	+++	++	++	+/– (side chain)	–
Sia	+++	+++	–	+++	+/– (side chain)	–
GlcA	+?	–	+++	+	–	–
SO_4	+	+	++++	+	–	–
P_i esters	Man-6-P	–	Xyl-P	–	M6P (in core)	–
O-Acetyl	Sia-OAc	Sia-OAc	–	Sia-OAc	–	–
Acyl	–	–	–	++++	+/– (on inositol)	–

[a]The relative distribution of the different monosaccharides and modifications in the commonly occurring glycoconjugates, indicated by a relative scale from + to ++++, is generally valid over many cell types. However, an uncommon monosaccharide may be commonly found in some cell types or glycoconjugate (e.g., most pituitary glycoprotein hormones have GalNAc as a major component of their *N*-linked oligosaccharides). Other symbols: +?, possible distribution; +/–, variably present; –, not identified to date.

ing uptake and incorporation) and type of glycoconjugate to be labeled. In most cases, the precursor will be a monosaccharide; for an oligosaccharide chain containing one or more ester modifications of sugars, it is also possible to use a precursor such as acetate, sulfate, or orthophosphate.

Factors affecting specificity and final distribution. In selecting a monosaccharide precursor, it should be noted that the distribution of monosaccharides among different types of vertebrate oligosaccharides is nonrandom (Table 12.2.3). It is also important to consider the metabolic pathways for uptake, activation, utilization, and interconversion of the various monosaccharides and their nucleotide sugars, which have been studied extensively (Fig. 12.2.4 and Table 12.2.4). The final distribution and specific activity of label from a given radiolabeled monosaccharide can be significantly affected by dilution from an unlabeled compound generated by endogenous pathways, the characteristics of the particular cell type being labeled, and the labeling conditions (Kim and Conrad, 1976; Yurchenco et al., 1978; Yanagishita et al., 1989). The exact position of the tritium label within a monosaccharide can affect the label's ultimate fate (Diaz and Varki, 1985). Interconversion between monosaccharides (e.g., conversion to glucose; Fig. 12.2.4), which can be expected to occur with prolonged labeling, will eventually result in labeling of non-oligosaccharide components. An exception is labeling with [2-^{3}H]mannose: this is extremely specific, because conversion of the labeled mannose can yield only [2-^{3}H]fucose or unlabeled fructose-6-phosphate. In the latter case, the label is lost as tritiated water, which is diluted into the pool of cellular water. Thus, in most instances, label from [2-^{3}H]mannose remains confined to mannose and fucose residues, regardless of how long the labeling continues.

Factors affecting uptake and incorporation. With radioactive amino acids, high-specific-activity labeling can be obtained by omitting the unlabeled molecule from the medium. Labeling with radioactive sugars or other oligosaccharide precursors, however, is usually less efficient. Thus, incorporation into the glycoconjugate of interest must be optimized empirically, taking into consideration the following factors:

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Experimental parameters. Amount of label, concentration of label in the medium, cell number, duration of labeling, and number of cell doublings during labeling are obviously important. The goal is to expose the maximum number of cells to the maximum amount of label in the minimum volume of medium for the longest period of time. Some of these factors are at odds with one another, and the correct balance must be tailored to the particular cell type and the question being investigated.

Competition with glucose. When labeling using a radioactive monosaccharide precursor, it is important to determine whether the monosaccharide competes with glucose for uptake. The high concentration of glucose in normal tissue culture medium (~5 mM) means that monosaccharides competing with glucose for active transport cannot be taken up very well. Thus, lowering glucose concentration can improve uptake. On the other hand, monosaccharides that do not compete with glucose seem to be taken up only by inefficient noncompetitive, passive mechanisms; thus, glucose concentration has little effect upon their already poor uptake. Experience indicates that glucosamine, galactosamine, galactose, and mannose can compete with glucose for uptake into most cells, whereas *N*-acetylglucosamine, *N*-acetylmannosamine, mannosamine, fucose, and xylose do not (Yurchenco et al., 1978). However, members of the glucose transporter family of gene products are tissue-specific in expression; a given cell type may express more than one,

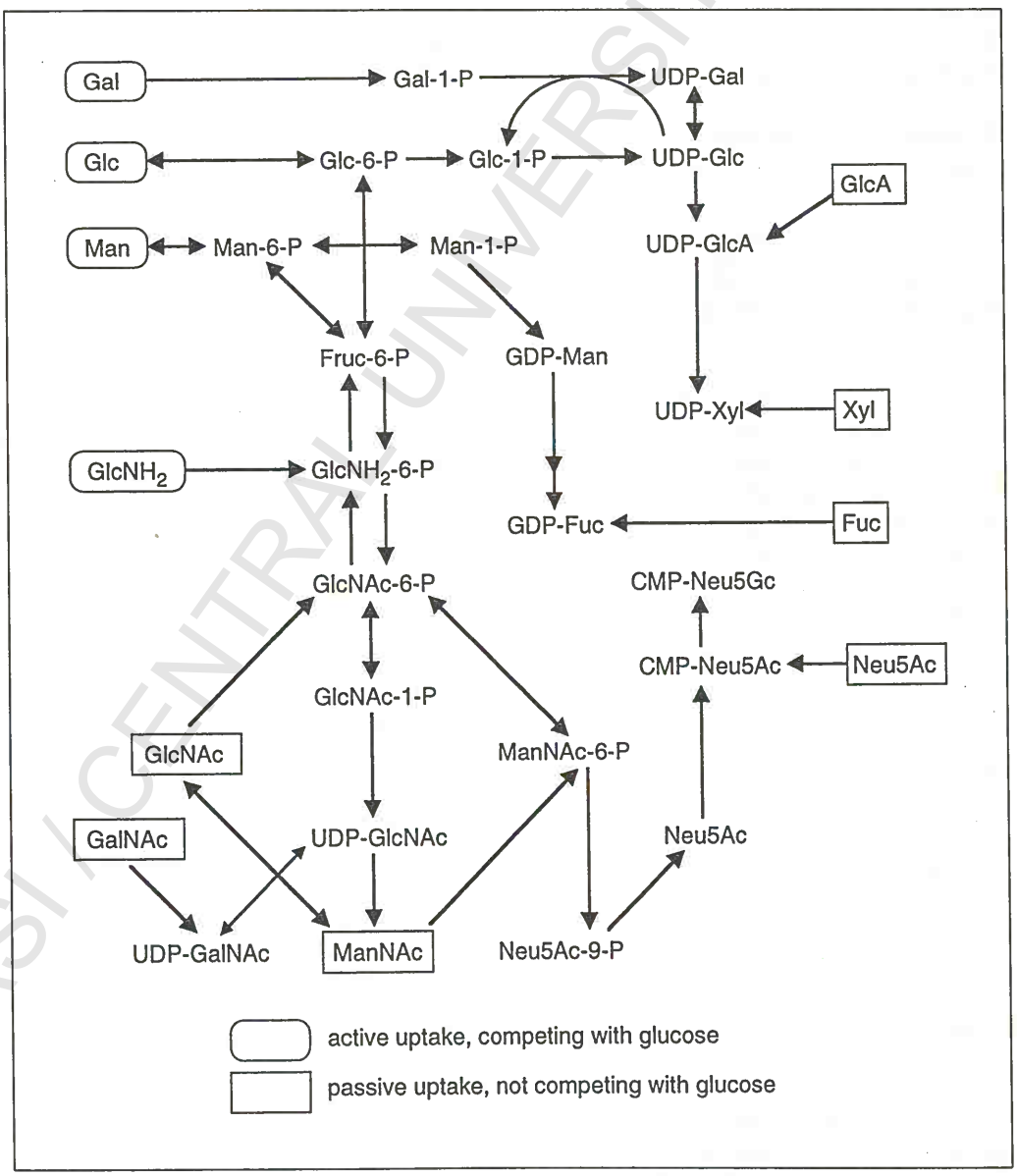

Figure 12.2.4 Cytosolic pathways for the interconversion of monosaccharides and their nucleotide sugar forms.

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Table 12.2.4 Cellular Monosaccharides Labeled with Radioactive Precursors[a]

Product identified as:	Radiolabeled precursor						
	[2-^{3}H]Man	[6-^{3}H]Gal	[1-^{3}H]Gal	[1-^{3}H]Glc	[6-^{3}H]GlcNH$_2$	[6-^{3}H]ManNAc	[6-^{3}H]GalNAc
[^{3}H]Man	+++	+/–	+/–	++	+/–	+/–	+/–
[^{3}H]Fuc	+++	+/–	+/–	++	+/–	+/–	+/–
[^{3}H]Gal	–	++++	++++	+++	+/–	+/–	+/–
[^{3}H]GlcA	–	–	++	++	–	–	–
[^{3}H]Glc	–	++	++	++++	+/–	+/–	+/–
[^{3}H]GlcNAc	–	+/–	+/–	++	++++	++	+++
[^{3}H]GalNAc	–	+/–	+/–	++	+++	+	++++
[^{3}H]Sia	–	–	–	++	++	+++	+/–

[a]The extent of conversion from the original monosaccharide into minor pathways will vary considerably, depending upon the cell type studied and the length of the labeling (longer time periods tend to result in more conversion into other monosaccharides). The extent of incorporation is indicated by a relative scale from + to ++++. Other symbols: +/–, variable low-level incorporation; –, not identified to date.

each with distinctive kinetic and stereospecific uptake properties (Gould and Bell, 1990). Thus, each monosaccharide must be tested in the specific cell type under study to see if it competes with glucose. A final point that should be considered is that in some cell types, lipid-linked oligosaccharide precursors may become altered when glucose-free medium is used (Rearick et al., 1981).

Other intracellular metabolic factors. Dilution of label by endogenously synthesized monosaccharides, pool size of individual monosaccharides and nucleotides, and flux rates between interconverting pathways can all affect the final specific activity and distribution of the label. If cells are cultured in labeling medium for prolonged periods of time, the medium may become depleted of glucose (Kim and Conrad, 1976; Varki and Kornfeld, 1982). The specific activity of the labeled sugar nucleotides in the cells may then actually rise as the medium glucose concentration falls below the K_m of the cellular glucose transporter(s). These factors must be considered in designing the labeling protocol, although they can be controlled only to a limited extent. The goal is to obtain sufficient label in the glycoconjugate of interest without significantly altering the metabolic state of the cell.

Metabolic labeling using nonmonosaccharide precursors. Acetylation, sulfation, and phosphorylation can significantly affect the behavior of oligosaccharides both in biological systems and during analysis. Labeled precursors such as [^{3}H]acetate, [^{35}S]sulfate, and [^{32}P]orthophosphate can be used to label these modified oligosaccharides. These precursors are expected to enter a variety of other macromolecules, and release or isolation of the labeled oligosaccharides with specific endoglycosidases is often required before further analysis. Double-labeling of both the modified oligosaccharide and the underlying sugar chain (e.g., with a ^{14}C- or ^{3}H-labeled monosaccharide precursor) can be quite useful in monitoring purification and in subsequent structural analysis. With [^{35}S]sulfate, labeling efficiency varies widely with cell type. In many cells, if the endogenous pool of the precursor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) is very small, the specific activity of the exogenously added [^{35}S]sulfate can be practically unaltered inside the cell (Yanagishita et al., 1989). In some cell types, however, the endogenous sulfate pool is constantly diluted by breakdown of the sulfur-containing amino acids methionine and cysteine (Esko et al., 1986; Roux et al., 1988). In this situation, reducing the cysteine and methionine concentrations in the medium can improve incorporation of label into macromolecules (Roux et al., 1988). Sulfate levels can also be affected by certain antibiotics (e.g., gentamycin) that are sulfate salts.

Determining the specific activity of incorporated label

In most instances, the precise specific activity of each monosaccharide pool need not be determined. If steady-state labeling is attempted, a plateau in the rate of incorporation of label per milligram of cell protein can be taken as a rough indication that a steady state has been reached. In some cases, however, the precise specific activity of a given monosaccharide may be of interest. Discussions of how this can be determined can be found in Kim and Conrad

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(1976), Yurchenco et al. (1978), Yanagishita et al. (1989), and Varki (1991). Endogenous glucose is the normal precursor for hexoses and hexosamines (see Fig. 12.2.4). Experimental manipulations that alter the concentration of glucose can thus alter the concentration of the internal pool of other hexoses and hexosamines. Exogenously added labeled hexosamines become diluted within the cell, making specific radioactivity in the cell lower than that of the starting material.

With the advent of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), measurement of monosaccharide levels in the low picomole range is now possible. Thus, it is now feasible to measure the specific activity of a labeled monosaccharide component of a purified oligosaccharide by acid hydrolysis of a portion of the oligosaccharide (Hardy et al., 1988) followed by HPAE-PAD. High-performance liquid chromatography (HPLC)–based methods for accurate measurement of low quantities of sulfate are also now available.

Troubleshooting

Ideally, the radiochemical purity of the precursor should be checked using an appropriate chromatographic procedure. As with all tissue culture work, cells should be checked for mycoplasma infection (Strober, 1991) before the experiments are carried out. If bacterial contamination poses a problem for long-term labeling experiments, appropriate antibiotics should be added to the medium.

Anticipated Results

Levels of incorporation will be highly variable, depending upon the cell type, the culture conditions, and the precursor used. If adequate incorporation into the glycoconjugate of interest is obtained, analyses can be carried out.

Time Considerations

Optimizing conditions for uptake and incorporation of label for each precursor and cell line combination (introductions to the first two protocols) may take one to two weeks, but should not be omitted: the time will be well-spent. The time required for the actual labeling protocols will vary widely depending on the goal of the experiment. Preparation of MDM can take an entire day, especially if some components must be prepared from scratch. If the amount required for a series of experiments is planned ahead of time, however, this need only be done occasionally. Reconstituting MDM takes up to 1 hr (assuming all the stock solutions are already available).

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Inhibition of N-Linked Glycosylation

Treatment of cells with inhibitors of the enzymes that synthesize N-linked oligosaccharide chains results in production of glycoproteins containing missing or altered chains. This approach is useful for examining potential functional role(s) of this class of oligosaccharides on specific proteins or intact cells. Table 12.3.1 lists the enzymes blocked by each inhibitor and the consequences; Figure 12.3.1 diagrams the early processing steps in the assembly of N-linked oligosaccharides, showing the enzymes blocked by each inhibitor except tunicamycin (which affects an earlier stage). This unit describes the use of these inhibitors to prevent N-linked glycosylation of proteins in cultured cells. First, the optimal concentration of inhibitor for the experiment (i.e., highest nontoxic concentration) is determined by monitoring [^{35}S]methionine incorporation as a measure of protein biosynthesis. The inhibitor's ability to inhibit oligosaccharide processing is then determined by analyzing cells labeled with [^{3}H]mannose using TCA precipitation or endo H digestion. A Support Protocol details a method for concentrating proteins by acetone precipitation. Although not specifically covered in this unit, these inhibitors can be used to examine specific glycoproteins that have been identified and/or purified (e.g., by immunoprecipitation, immunoblotting) in a scheme established by the investigator. As an example, several of the inhibitors render the oligosaccharide chains on a glycoprotein susceptible to removal by endo H, and the change in size of the glycoprotein is frequently large enough to be detected by SDS-PAGE (*UNIT 10.1*). Thus, radiolabeled samples (e.g., labeled with [^{35}S]Met, ^{125}I) from inhibitor-treated cells can be purified, and the effects of endo H digestion utilized to determine the effect of the inhibitor on oligasaccharide processing.

NOTE: All media and solutions should be made with Milli-Q-purified (or equivalent) water. All media and equipment coming into contact with cells should be sterile. Culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator.

Materials

Cultured cell line, either adherent or suspension
Complete culture medium
Inhibitor of N-linked glycosylation (one or more of the following: tunicamycin, deoxynojirimycin, castanospermine, deoxymannojirimycin, or swainsonine; see recipe for inhibitor stock solutions and Table 12.3.2)
Solvent used for making inhibitor solution (see recipe)
Multiply deficient medium (MDM; *UNIT 12.2*) supplemented with 200 µM glucose
[^{3}H]mannose (5 to 20 Ci/mmol)
Phosphate-buffered saline (PBS; *APPENDIX 2E*), ice cold
Lysis buffer (see recipe)
Sepharose, activated
0.5 U/ml endoglycosidase H (endo H) and endo H digestion buffer (see recipe)
20% (w/v) SDS (*APPENDIX 2E*)
Sephacryl S-200 column (*UNIT 8.3*), calibrated and equilibrated in 25 mM ammonium formate/0.1% SDS
25 mM ammonium formate/0.1% (w/v) SDS

24-well tissue culture plate
100-mm tissue culture plates
Disposable plastic scraper or rubber policeman
Centrifuges and appropriate rotors: low-speed and ultracentrifuge
1.5-ml, 15-ml, or 50-ml conical polypropylene centrifuge tube

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Table 12.3.1 Inhibitors of Enzymes that Synthesize N-linked Oligosaccharides

Type of inhibitor	Target enzyme	Effect on oligosaccharide structure	Effective concentration range
Glycosylation inhibitor			
Tunicamycin	GlcNAc transferase	Prevents assembly of GlcNAc-PP-dolichol and thus assembly of $G_3M_9GlcNAc_2$-PP-dolichol[a]; glycosylation of Asn residues does not occur; proteins migrate faster on SDS-PAGE and generally show less size heterogeneity; proteins will not shift to a faster mobility on SDS-PAGE after PNGase F treatment	0.5-10 μg/ml
Processing inhibitors			
Deoxynojirimycin Castanospermine	Glucosidase I and/or II	Prevents removal of first glucose residue, thereby inhibiting any further processing of the oligosaccharide chain; proteins may migrate with a larger or smaller size on SDS-PAGE, depending on extent of processing that normally occurs; sensitive to endo H[b]	0.5-20 mM 1-50 μg/ml
Deoxymanno-jirimycin	α-mannosidase I	Prevents removal of mannose residues on the α1-3 arm of the high mannose structure, thereby blocking the activity of GlcNAc T I and thus α-mannosidase II; proteins generally run as a smaller size on SDS-PAGE, depending on size of the normal oligosaccharide; structures remain sensitive to endo H[c]	1-5 mM
Swainsonine	α-mannosidase II	Prevents removal of mannose residues on the α1-6 arm of the high mannose structure, preventing the activities of GlcNAc T II and V (which add GlcNAc to the α1-6 mannose residue); addition of GlcNAc, galactose, and sialic acid to the α1-3 mannose can occur normally, although the structures remain sensitive to endo H digestion	1-10 μg/ml

[a]Abbreviations: $G_3M_9GlcNAc_2$-PP-dolichol, $Glc_3Man_9GlcNAc$-PP-dolichol; GlcNAc T I, II, V, *N*-acetylglucosaminyltransferase I, II, V; PNGase F, peptide N:glycosidase F.

[b]Mature processed oligosaccharide structures cannot be cleaved from the protein by endoglycosidase H; structures retaining mannose residues on the α1-6 arm can be removed, generally resulting in a difference in size easily detected by SDS-PAGE.

[c]Endomannosidases can bypass this block.

Additional reagents and equipment for metabolic radiolabeling of glycoconjugates (*UNIT 12.2*), gel-filtration chromatography (*UNIT 8.3*), and one-dimensional SDS-PAGE (*UNIT 10.1*)

Determine optimal inhibitor concentration

1. For each inhibitor tested, set up 15 wells of cells in a 24-well tissue culture plate by splitting the cells (using trypsinization for adherent cells or dilution for suspension cells) into 1.8 ml final volume per well of complete medium. Incubate 24 hr.

   The ratio of splitting should be designed to yield confluent (but not overgrown) cultures by the third day (i.e., 48 hr after splitting).

2. While cells are incubating, make a series of 1:1 (v:v) dilutions of inhibitor(s) as follows. Place the volume of inhibitor stock solution indicated in Table 12.3.2 in a sterile microcentrifuge tube and dilute to 800 μl final volume with complete culture medium. Transfer 400 μl to a second tube and dilute with 400 μl complete culture medium. Repeat for a total of seven tubes. As a control, prepare an identical series of dilutions of the solvent used for making the inhibitor solution.

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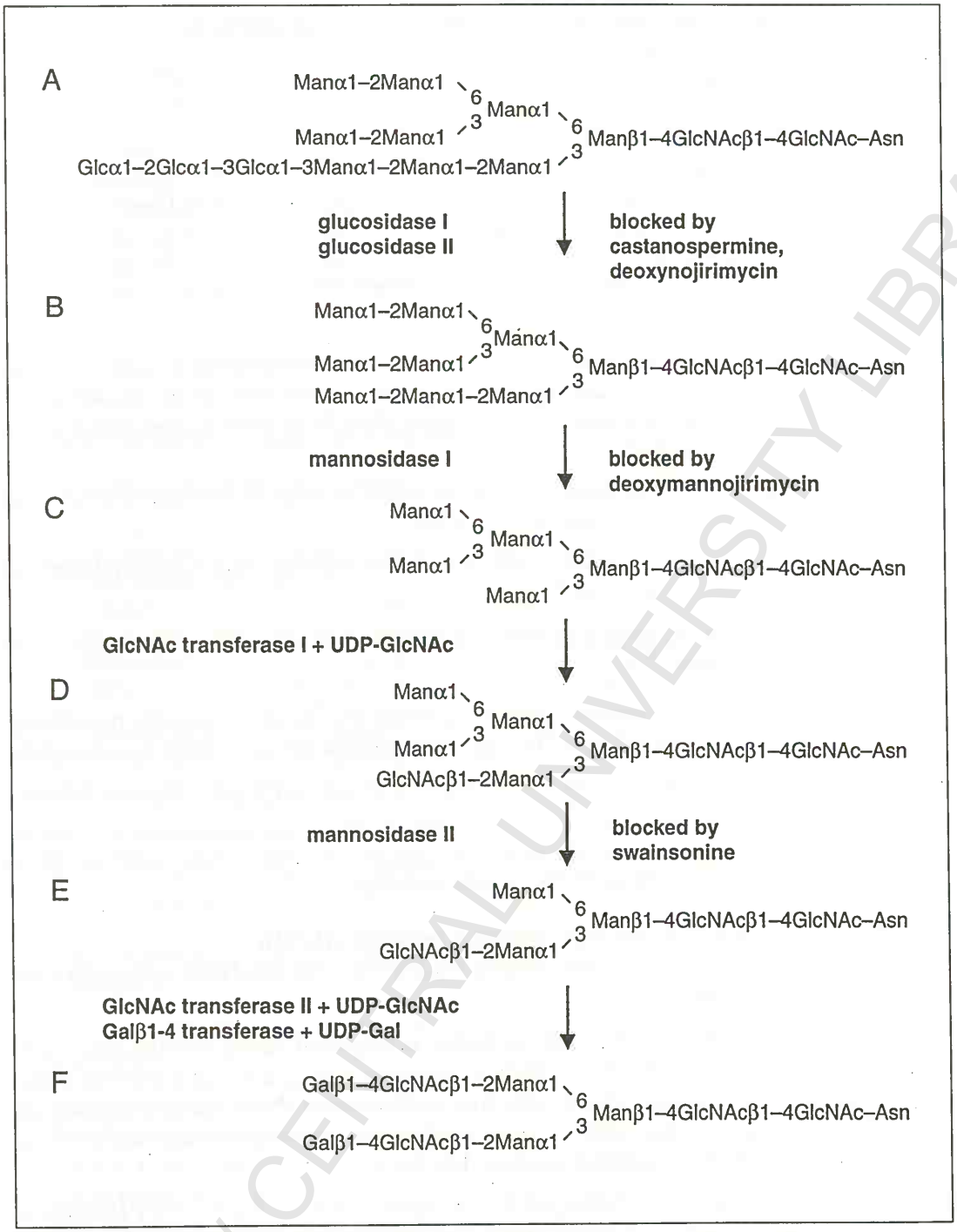

Figure 12.3.1 Structures of early processing intermediates in the assembly of N-linked oligosaccharides. Structure A is initially assembled on the lipid carrier dolichol phosphate, a process blocked by tunicamycin, and then transferred to the Asn residue. The initial trimming glucosidases I and II convert A into B; these glucosidases are inhibited by castanospermine and deoxynojirimycin. Deglucosidation is followed by removal of the outer four mannose residues by mannosidase I (inhibited by deoxymannojirimycin) to form C. Removal of these mannose residues is the signal for the first *N*-acetylglucosaminyltransferase (GlcNAc transferase I) to form D. Structure D, but not C, is a substrate for mannosidase II, resulting in the formation of E, which can be further extended into bi-, tri-, and tetraantennary structures (sialylated and neutral) by a series of *N*-acetylglucosaminyltransferases, galactosyltransferases, and sialyltransferases plus the appropriate sugar-nucleotide donors.

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Table 12.3.2 Range of Inhibitor Concentrations for Dilution Series

Inhibitor	Stock solution	Stock added to first dilution (µl)	Concentration range
Tunicamycin	1 mg/ml	80	0.15-10 µg/ml
Swainsonine	1 mg/ml	80	0.15-10 µg/ml
Deoxynojirimycin	400 mM	200	0.15-10 mM
Deoxymannojirimycin	400 mM	200	0.15-10 mM
Castanospermine	400 mM	200	0.15-10 mM

3. Add 200 µl from each tube (from step 1) to a well of the tissue culture plate containing cells (2 ml final). Add 200 µl complete culture medium alone to the remaining well (as a zero-inhibitor control point). Incubate plate 24 hr. Store unused inhibitor-containing media at 4°C.

   If the inhibitor is tunicamycin, dilutions should be prepared fresh each day, as it may precipitate out of solution during storage.

4. Perform short-term labeling of cells with [^{35}S]methionine using 0.2 mCi/ml label and 1.8 ml final volume per well.

5. Add to wells the remaining 200 µl of each dilution of inhibitor (from step 2) and incubate a further 4 hr.

6. Harvest cells and determine the incorporation of [^{35}S]methionine into macromolecules by TCA precipitation. Plot label incorporation versus inhibitor concentration.

   CAUTION: *Safety precautions for disposing of radioactive waste should be followed.*

   Comparing incorporation by cells incubated with and without inhibitor will indicate the maximum concentration of inhibitor that does not inhibit protein synthesis. This is the optimal concentration for the following steps.

Label cells in the presence of N-linked glycosylation inhibitor

7. Split a new cell sample into complete medium in a 100-mm tissue culture plate and incubate 24 hr.

8. Wash the cells using the same procedure as employed during labeling (step 4). To each plate, add sufficient MDM to cover the cells (e.g., 5 ml for a 100-mm plate). Add inhibitor to the optimal concentration determined above. Set up a control plate containing the same quantity of stock solution solvent but no inhibitor. Add [^{3}H]mannose to 0.02 to 0.1 mCi/ml. Incubate 4 to 12 hr.

   The duration of incubation will depend on how well the cells label with [^{3}H]mannose. The amount of [^{3}H]mannose used will depend on the cell line examined. However, for most cells, a single 100-mm plate with 5 ml media incubated with 0.02 mCi/ml [^{3}H]mannose for 6 hr should yield sufficient radioactive material for the analyses described below. "Glucose-free" MDM is described in UNIT 12.2*; in fact, cells should not be labeled in the total absence of glucose, as this causes alterations in glycan processing. For this reason, the glucose concentration should be kept above 200 µM.*

   For metabolic labeling, preincubation of the cells with inhibitor is not essential. However, a 24-hr preincubation with inhibitor is recommended for the cell-surface expression of a sufficient population of glycoproteins with altered glycan structures.

9. Harvest the cells by scraping with a disposable scraper or rubber policeman (for adherent cells) or centrifugation (for suspension cells) into ice-cold PBS.

   CAUTION: *Safety precautions for disposing of radioactive waste should be followed.*

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Measure incorporated macromolecular radioactivity

10. If tunicamycin was used as the inhibitor, determine the amount of incorporated macromolecular radioactivity by TCA precipitation and proceed to step 18. If another inhibitor was used, carry out steps 11 to 18.

    The amount of radioactivity incorporated into macromolecules reflects the effect of the inhibitor(s) on oligosaccharide biosynthesis: the presence of inhibitor should block incorporation of [^{3}H]mannose into macromolecules.

11. Wash the cells twice by resuspending the pellet in ice-cold PBS, centrifuging, and removing the supernatant. Resuspend the pellet in lysis buffer (5×10^7 cells/ml) and incubate 1 hr at 4°C.

12. Centrifuge the lysate 10 min at 3000 × *g*, 4°C, to remove nuclei and save the supernatant.

13. Centrifuge the supernatant 1 hr at 100,000 × *g*, 4°C, and save the supernatant.

    Supernatants may also be prepared by microcentrifugation (10,000 × g) for 30 min.

    The supernatant must be used within several days or stored at –70°C. The length of storage is limited by autoradiolysis and the half-life of the isotope. ^{3}H- and ^{14}C-labeled samples can often be stored frozen for years. Storage of ^{125}I-labeled samples is usually limited to 1 to 2 months because of autoradiolysis. The usefulness of ^{35}S-labeled samples is usually limited to 6 months because of half-life. Repeated freezing and thawing may disrupt antigenic determinants and dissociate some protein complexes, especially those that are noncovalently associated.

14. Divide each sample into two equal aliquots in conical polypropylene centrifuge tubes and precipitate protein with acetone (see Support Protocol).

    Use 1.5-ml, 15-ml, or 50-ml centrifuge tubes, depending on the quantity of sample (8 vol acetone must be added).

15. Resuspend pellets in 20 to 40 µl endo H digestion buffer per 10^7 cells and boil 10 min to inactivate endogenous hydrolases.

16. Add 5 µl endo H per 100 µl sample to one tube of each sample pair and incubate both overnight at 37°C.

17. Add 1/10 vol of 20% (w/v) SDS to each sample and boil 3 to 5 min to terminate the digestion.

18. Apply one sample at a time to a calibrated Sephacryl S-200 column equilibrated in 25 mM ammonium formate/0.1% SDS, in a sample volume that is <5% of the bed volume. Collect the eluate and use a scintillation counter to measure the radioactivity associated with glycoproteins (eluting in the void volume of the column, V_o) and with free oligosaccharides (eluting after V_o) for each of the four samples. Wash column with 2 to 4 vol 25 mM ammonium formate/0.1% SDS between sample applications.

    In the absence of endo H digestion, all the radioactivity from both the inhibitor-treated and untreated samples should elute in the V_o. Elution of any radioactive material after the V_o may indicate that the wash steps following acetone precipitation did not completely remove low-molecular-weight contaminants present in the cell lysate.

    For samples digested with endo H, elution profiles should demonstrate a marked increase in the amount of endo H-releasable radioactivity in samples from inhibitor-treated cells relative to treated cells. For samples not treated with inhibitor, the oligosaccharides cleaved from the peptide backbone by endo H represent immature oligosaccharides still being processed in the Golgi apparatus and a small population of endo H-sensitive structures found on some mature glycoproteins. For samples treated with inhibitor, a much higher percentage of the total radioactivity should be

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released, as the inhibitors prevent the processing of the oligosaccharides into mature oligosaccharide structures (Fig. 12.3.1) and the underprocessed oligosaccharides contain more mannose residues than the processed ones. Theoretically, 100% of the radioactivity should be releasable by endo H digestion of the inhibitor-treated samples (see Critical Parameters), although in reality no inhibition is ever complete.

To save time, analyze only the first and last tubes of the control cells in this fashion, i.e., those not treated at all and those treated with the highest concentration of the inhibitor solvent (from step 2 above). If there is no difference in the results from these two groups of cells, it is reasonable to assume that those cells treated with lower concentrations of the inhibitor solvent will yield the same results. However, if the solvent is found to be toxic, then the cells exposed to lower concentrations will need to be examined to find the maximally tolerated concentration of inhibitor solvent.

19. If desired, and if methods are available for purifying and identifying a specific glycoprotein from cells radiolabeled with [^{35}S]methionine, examine the effect of a given inhibitor by SDS-PAGE and autoradiography.

    Each N-linked carbohydrate chain contributes ~2000 to 4000 Da to a protein's mass, so synthesis of a protein in the presence of tunicamycin will result in faster mobility on SDS-PAGE. The effect of other inhibitors can be demonstrated by an increased sensitivity to digestion by endo H, which is also detectable as an increase in migration rate in SDS-PAGE.

SUPPORT PROTOCOL

ACETONE PRECIPITATION

Acetone precipitation is a useful step for concentrating proteins and for exchanging them from one buffer to another. As little as 10 ng of protein can be precipitated successfully.

Additional Materials (also see Basic Protocol)

100% acetone (HPLC or ACS grade), –20°C

1. Place the protein solution on ice, add 8 vol acetone (–20°C), mix gently, and precipitate at –20°C overnight.

   If the sample volume is too large to be conveniently diluted with 8 vol, concentrate it by lyophilizing and resuspending in a smaller volume. If the sample contains NP-40 or Triton X-100 detergent, however, there is a limit to how much it can be concentrated, as high concentrations of detergent (e.g., >1%) will partition out of acetone solutions, appearing as an oily pellet after centrifugation (step 2).

   If a large amount of protein is present (i.e., a precipitate is readily visible after the acetone is added), it can be precipitated after standing at –20°C for only a few hours instead of overnight. Leaving the material in acetone for longer than 1 day should be avoided, as it may become increasingly difficult to redissolve the protein.

2. Collect the precipitate by centrifuging 15 min at 3000 × *g*, 4°C (a small pellet should be visible). Carefully invert the tube and pour out the acetone into a clean tube.

   If the pellet is oily due to the presence of detergent, it should be extracted by adding 85% (v/v) acetone (–20°C), mixing, incubating at –20°C for >1 hr, and recentrifuging.

3. Centrifuge the tube containing the pellet briefly to concentrate the remaining acetone and remove it with a micropipettor. Allow pellet to dry until moist but not powder-dry, and resuspend in an appropriate quantity of the desired solvent.

   Care must be taken in drying the pellet, as great difficulty may be encountered in resuspending some proteins if the pellet dries completely. This insolubility depends on both the individual protein and the total amount present.

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REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Endo H digestion buffer

15 µl 0.5 M sodium citrate, pH 5.5
80 µl water
1 µl 100 mM PMSF in absolute ethanol
Prepare just before use (PMSF is unstable in water)

This is sufficient for 5 µl of 0.5 U/ml endo H (100 µl digestion volume). The PMSF prevents proteolysis. Nonionic detergent is not required.

Inhibitor stock solutions

Dissolve castanospermine (mol. wt. 189.2), 1-deoxymannojirimycin (mol. wt. 199.6), or 1-deoxynojirimycin (mol. wt. 163.2) in water to 400 mM; dissolve swainsonine (mol. wt. 173.2) in water to 1 mg/ml. Sterilize by filtration through a 0.2-µm filter. Store frozen at –20°C (stable indefinitely).

Dissolve tunicamycin (mol. wt. 840) in dimethylsulfoxide (DMSO), dimethylformamide (DMF), 95% ethanol, or 25 mM NaOH to 1 mg/ml. Store frozen at –20°C (stable ~1 yr).

If NaOH is used, store stock solution in a small tube that is tightly capped to prevent adsorption of carbon dioxide, which will lower the pH. Tunicamycin's solubility in neutral aqueous solution is <1 mg/ml (attempting to make such a solution will produce a visible precipitate).

Lysis buffer

1% Triton X-100
1 mM iodoacetamide (freshly prepared)
Aprotinin (0.2 trypsin inhibitor U/ml)
1 mM PMSF (add fresh from 100 mM stock in absolute ethanol)
Prepare in TSA solution (see recipe)

Tris/saline/azide (TSA) solution

0.01 M Tris·Cl, pH 8.0
0.14 M NaCl
0.025% NaN_3

CAUTION: *Sodium azide (NaN_3) is poisonous; wear gloves.*

COMMENTARY

Background Information

Assembly of N-linked oligosaccharide chains involves the sequential action of several glycosidases and glycosyltransferases (reviewed by Hubbard and Ivatt, 1981, and Kobata and Takasaki, 1992). Growth of cells in the presence of inhibitors of these enzymes produces glycoproteins with underprocessed or missing oligosaccharide chains. Depending on the protein, these changes may alter biological functions and/or rates of transport, secretion, or turnover (Elbein, 1987; McDowell and Schwarz, 1988).

N-linked glycosylation begins with the assembly of the lipid-linked precursor glucose$_3$-mannose$_9$*N*-acetylglucosamine$_2$-pyrophosphate-dolichol ($Glc_3Man_9GlcNAc_2$-PP-dolichol; similar to structure A, Fig. 12.3.1). Assembly of this precursor requires *N*-acetylglucosamine-pyrophosphate-dolichol (GlcNAc-PP-dolichol), whose formation is blocked by tunicamycin (Tkacz and Lampen, 1975). Thus, the presence of tunicamycin results in proteins missing some or all of their N-linked side chains; however, those that are added due to incomplete inhibition are processed normally. Tunicamycin may also inhibit protein synthesis; not all proteins will be affected to the same degree. Tunicamycin also blocks the assembly of type II keratan sulfate chains, because these glycans also utilize GlcNAc-PP-dolichol (Hart and Lennarz,

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1978). Glycolipid biosynthesis may also be inhibited, although the mechanism underlying this has not been established (Yusuf et al., 1983; Guarnaccia et al., 1987).

The glycosidase inhibitors castanospermine, deoxynojirimycin, deoxymannojirimycin, and swainsonine are considerably less toxic than tunicamycin. Castanospermine inhibits glucosidase I, and deoxynojirimycin inhibits both glucosidases I and II (Fig. 12.3.1; Pan et al., 1983; Saunier et al., 1982). In some studies, the metabolic block produced by these two drugs is not complete, yielding a mixture of normally processed and underprocessed structures. More recently, an endo-α-D-mannosidase has been described that cleaves the tetrasaccharide Glc_3-Man_1 from $Glc_3Man_9GlcNAc_2$, effectively bypassing the block produced by the glucosidase inhibitors (Lubas and Spiro, 1987). Although the question has not been examined fully, the level of this endo-α-D-mannosidase may vary between different cell lines.

Deoxymannojirimycin and swainsonine are inhibitors of mannosidases I and II, respectively (Fig. 12.3.1; Fuhrmann et al., 1984; Tulsiani et al., 1982). By inhibiting mannosidase I, deoxymannojirimycin prevents addition of the α1-2 *N*-acetylglucosamine (GlcNAc) residue required to produce structure D, Figure 12.3.1, which is a necessary step for the action of mannosidase II. Thus, deoxymannojirimycin prevents addition of any GlcNAc residues, blocking galactosylation and sialylation as well. Swainsonine blocks mannosidase II. However, it does not prevent the single GlcNAc residue (structure D, Fig. 12.3.1) from being galactosylated and sialylated, resulting in hybrid structures (Tulsiani and Touster, 1983).

Some of the glycosidase inhibitors are also active against lysosomal enzymes, although the significance of this effect in tissue culture cells has been little explored. Deoxynojirimycin can inhibit synthesis of the lipid-linked precursor in cell-free extracts (Romero et al., 1985); this also has not been thoroughly investigated with intact cells. No information is available on the metabolism or half-lives of these inhibitors in cultured cells.

Critical Parameters and Troubleshooting

The two most critical variables are duration of exposure and inhibitor concentration. The treatment times suggested in this unit (24 hr) will permit a cell to replace most of its endogenous glycoproteins with glycoproteins synthesized in the presence of inhibitor. For proteins that turn over slower or faster than average, longer or shorter incubation times may be appropriate. If pulse-chase studies with radiolabeled sugar or amino acid precursors (*UNIT 12.2*) are being carried out, it is necessary to incubate cells in inhibitor for only 1 to 2 hr before adding label.

N-linked glycosylation inhibitors may be toxic to the cells, as indicated by decreased cell viability and/or reduced protein synthesis. The concentration of tunicamycin that can be used is generally limited by its toxicity, which may be considerable in some cell lines (more so with tumor cells than nontransformed cells). Other glycosidase inhibitors are usually not toxic in the concentration ranges suggested in Table 12.3.1 (the highest concentrations listed represent those used in most published studies).

It is important to realize that in a given cell line, not all glycoproteins will be affected to the same extent by a given inhibitor. Moreover, it is possible that not all sites on the same glycoprotein will be affected equally. These caveats should be kept in mind when interpreting the results from studies employing these inhibitors.

Failure to observe an effect from an inhibitor may indicate that concentration of inhibitor was too low and/or length of exposure was too short. Cell lines differ in their susceptibility to these compounds.

Anticipated Results

These experiments will demonstrate the chosen inhibitor's toxicity, as assessed by protein synthesis, and its ability to alter oligosaccharide biosynthesis, as assessed by total [^{3}H]mannose incorporation (for tunicamycin) or endo H release of the oligosaccharides (for the other inhibitors listed in Table 12.3.1).

Time Considerations

Setting up the initial round of cell cultures for determining the optimal inhibitor concentration takes 2 hr, after which they are incubated 24 hr. On the second day, the dilution series of medium containing inhibitor is set up, the cells are transferred to these media (<1 hr), and the plates incubated 24 hr. On the third day, the incubation of the cells with the radiolabeled precursor is set up (~4 hr); the actual incubation takes 4 to 16 hours. Following incubation, the cells are assayed for radiolabel incorporation by TCA precipitation (2 to 4 hr).

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The main part of the experiment follows a similar time course if the inhibitor used is tunicamycin. If a different inhibitor is used, analysis of endo H susceptibility by Sephacryl S-200 chromatography will take ~1 day, including the overnight incubation.

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Useful general review.

McDowell, W. and Schwarz, R.T. 1988. See above.

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Hubbard, S.C. and Ivatt, R.J. 1981. See above.

Good review of early work on processing pathway.

Kobata, A. and Takasaki, S. 1992. See above.

Good review of recent work on some of the complexities in oligosaccharide processing.

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Endoglycosidase and Glycoamidase Release of N-Linked Oligosaccharides

Chemical treatments that remove sugar chains from glycoproteins are often extremely harsh and can completely destroy the protein and/or cleave labile groups from the carbohydrate chains. Endoglycosidases and glycoamidases cleave intact oligosaccharides from proteins under mild conditions that preserve the structures of both the oligosaccharide and the protein (see Fig. 12.4.1). The enzymes described in the basic protocols—endoglycosidase H (Endo H), endoglycosidase F_2 (Endo F_2), and peptide:*N*-glycosidase F (PNGase F)—are the enzymes most commonly used for digestion of intact glycoproteins and glycopeptides. Endo H cleaves high-mannose-type and hybrid oligosaccharide chains, while Endo F_2 cleaves biantennary complex-type oligosaccharides. PNGase F has the broadest specificity of the three; it cleaves tri- and tetraantennary complex-type chains as well as those cleaved by the other two enzymes (Fig. 12.4.2). In some instances, the specificities of these enzymes can be used to determine the number and structure of oligosaccharide chains and identify the cellular compartments the protein of interest has encountered. PNGase F is the preferred enzyme for complete removal of N-linked oligosaccharide chains, whereas sequential digestion with Endo H, Endo F_2, and PNGase F is used to differentiate high-mannose, biantennary, and tri- or tetraantennary chains. Either the deglycosylated protein or the liberated oligosaccharides can be studied following digestion. Although not every N-linked oligosaccharide chain is sensitive to the enzymes described here, these enzymes provide the best way to gently remove them from glycoproteins. These digestion protocols can be applied to small sub-nanogram amounts of radiolabeled proteins, large amounts of total cellular glycoproteins, soluble purified glycoproteins, or solubilized immunoprecipitates. The purified enzymes, from natural sources or recombinant expression systems, are commercially available.

The basic protocols describe the reaction conditions for Endo H, Endo F_2, and PNGase F. These digestions are designed for either complete or selective release of N-linked oligosaccharides from glycoproteins. In some cases, this is done to estimate the number or type of sugar chains present on one or more proteins. In other cases, digestion is performed to prepare the oligosaccharide chains for further analysis. The susceptible bonds of many oligosaccharides are not exposed in native proteins but become exposed and susceptible to enzymatic cleavage when proteins are denatured. Reversible [sodium thiocyanate (NaSCN)] or irreversible [sodium dodecyl sulfate (SDS)] denaturation of proteins can be used. Complete oligosaccharide removal starts with denaturation of solubilized glycoproteins in an appropriate buffer followed by digestion with the enzyme. The deglycosylated protein may be analyzed by one- or two-dimensional gel electrophoresis (*UNITS 10.1-10.4*) or the released sugar chains separated from the protein by gel-filtration chromatography (*UNIT 8.3*). When proteins are biosynthetically labeled with a radioactive amino acid, mobility changes may be detected by autoradiography of polyacrylamide gels. Immunoblotting may be used to detect nonradioactive proteins. For these protocols, the peptide or the oligosaccharide chains are assumed to be radiolabeled; however, the methods can be applied to unlabeled material if larger amounts are available for analysis (*UNIT 12.1*).

The applications described in the two support protocols employ these enzymes alone or as part of a sequential digestion. Support Protocol 1 describes how to use partial digestions with one enzyme or sequential digestions with different enzymes to estimate the number and/or types of N-linked carbohydrate chains on a protein. Support Protocol 2 describes sample preparation and digestion followed by gel-filtration chromatography to recover the released radiolabeled oligosaccharide chains for subsequent analysis.

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These procedures are ideally suited for analyzing small amounts of labeled proteins such as those from solubilized immunoprecipitates or cell lysates. If the exact amount of protein in a sample is not known, this should not affect digestion, as the enzyme is usually in great excess.

BASIC PROTOCOL 1

ENDOGLYCOSIDASE H DIGESTION

Endo H cleaves N-linked oligosaccharides between the two *N*-acetylglucosamine (GlcNAc) residues in the core region of the oligosaccharide chain (Figs. 12.4.1 and 12.4.2) on high-mannose and hybrids (but not complex-type) oligosaccharides that are free or bound to protein. The limited substrate specificity of this enzyme makes it useful for diagnosing the presence of these types of chain on mature proteins and for kinetic analysis of the conversion of high-mannose to complex-type oligosaccharides on biosynthetically labeled proteins as they move through the Golgi apparatus. In this protocol, a fully denatured protein is digested with Endo H to obtain complete release of sensitive oligosaccharides.

Materials

Protein sample
0.1 M 2-mercaptoethanol (2-ME)/0.1% (w/v) SDS (ultrapure electrophoresis grade; make fresh)
0.5 M sodium citrate, pH 5.5
10% (w/v) phenylmethylsulfonyl fluoride (PMSF) in isopropanol
0.5 U/ml Endo H (natural or recombinant; Genzyme, Oxford GlycoSystems, or Boehringer Mannheim)
30° and 100°C water baths

Additional reagents and equipment for estimating the number of N-linked oligosaccharide chains (see Support Protocol 1) or digesting and recovering the chains (see Support Protocol 2)

1. Place ≤25 µl of protein sample containing 15 to 20 µg protein in a microcentrifuge tube. Add 25 µl of 0.1 M 2-ME/0.1% SDS and heat denature 3 to 5 min at 100°C. Cool and microcentrifuge briefly to collect condensed droplets into bottom of tube.

   An immunoprecipitate or lyophilized sample is preferred so that the volume of the sample itself is small. If the estimated amount of protein exceeds 20 µg, use at least a 1.2- to 1.5-fold weight excess of SDS over protein to assure complete denaturation.

   Protein solubilization in nonionic detergents is not always sufficient to completely expose all susceptible cleavage sites. Only strong denaturation exposes all sites for maximum cleavage. If the protein was first precipitated with acetone (see Support Protocol 2), this can be removed during the heating step; alternatively, the protein can be resuspended in a small amount of water by sonication and then lyophilized prior to the SDS denaturation. The sample may also be incubated in 2.0 M NaSCN to denature the protein and then diluted to keep the final concentration of NaSCN less than 0.5 M during enzyme digestion.

2. Place an aliquot of the solubilized, denatured protein sample in a clean microcentrifuge tube for a control. To the remaining sample, add the following in order:

   15 µl 0.5 M sodium citrate, pH 5.5
   75 µl H_2O
   5 µl 10% PMSF (in isopropanol)
   5 µl 0.5 U/ml Endo H.

   Add the same reagents to the control tube, substituting water for enzyme.

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The amount of material used in the control will depend on the experiment and can be from 1% to 50% of the sample.

The PMSF prevents proteolysis. Nonionic detergent is not required to prevent inactivation of Endo H as long as high-purity SDS is used.

The amount of water can be varied. Other solutions (or an increased amount of sample) may be substituted for water if needed, but avoid potassium buffers because they precipitate SDS as a potassium salt. The reaction volume can be scaled up if required, but in nearly all cases Endo H will be in vast excess of what is required. Endo H can be used to deglycosylate hundreds of milligrams of glycoprotein for analysis of the protein or the released sugar chain.

3. Incubate overnight at 30° to 37°C.

   Some inactivation of the enzyme occurs at 37°C; however, it is present in sufficient excess that incubation can generally be carried out successfully at this temperature.

4. Inactivate Endo H by heating 5 min at 100°C.

5. Estimate the number of N-linked oligosaccharide chains on the protein (see Support Protocol 1) and/or recover and analyze these chains (see Support Protocol 2). Store protein sample at –20°C.

ENDOGLYCOSIDASE F_2 DIGESTION

BASIC PROTOCOL 2

Endo F_2, like Endo H, cleaves between the two GlcNAc residues in the chitobiose core (Figs. 12.4.1 and 12.4.2). It preferentially releases biantennary complex-type oligosaccharide chains from glycoproteins, but does not cleave tri- or tetraantennary chains. In this protocol, a glycoprotein sample is denatured and treated with Endo F_2 to release biantennary oligosaccharide chains.

Materials

Protein sample
0.1 M 2-mercaptoethanol (2-ME)/0.5% (w/v) SDS (make fresh)
0.5 M sodium acetate, pH 5.0 (*APPENDIX 2E*)
0.1 M 1,10-phenanthroline in methanol
10% (w/v) nonionic detergent: Nonidet P-40 (NP-40),
3-(3-cholamidopropyl)-dimethylammonio-1-propane sulfonate (CHAPS), or
n-octyl-β-D-glucoside
200 mU/ml Endo F_2 (Genzyme)

Additional reagents and equipment for estimating the number of N-linked oligosaccharide chains (see Support Protocol 1) or digesting and recovering the chains (see Support Protocol 2)

1. Place ≤10 µl of protein sample containing 30 to 50 µg protein in a microcentrifuge tube. Add 10 µl of 0.1 M 2-ME/0.5% SDS and heat denature 3 to 5 min at 100°C. Cool and microcentrifuge briefly to collect condensed droplets into bottom of tube.

   If more protein is digested, more SDS should be used—at least at 1.2- to 1.5-fold (w/w) excess over the estimated amount of protein.

2. Place an aliquot of the solubilized denatured protein sample in a clean microcentrifuge tube for control. To the remaining sample, add the following in order (19 µl total):

   10 µl 0.5 M sodium acetate, pH 5.0
   3 µl 1,10-phenanthroline in methanol
   5 µl 10% nonionic detergent
   1 µl 200 mU/ml Endo F_2.

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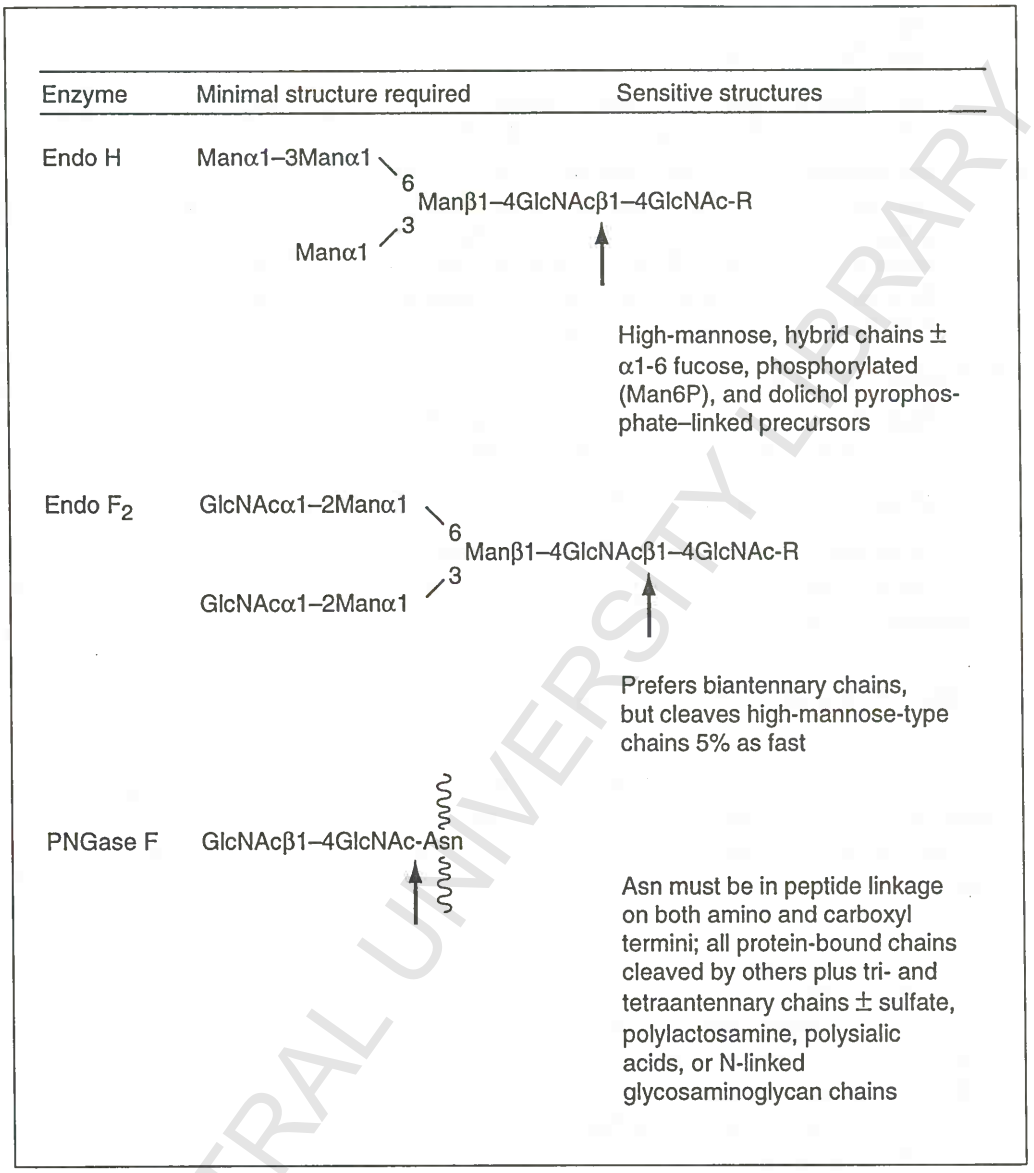

Figure 12.4.1 Sugar chains cleaved by endoglycosidases and glycoamidases. R, H, OH, Asn, or protein; Man, mannose; GlcNAc, *N*-acetyglucosamine; Man6P, mannose-6-phosphate; Asn with squiggles, Asn within a chain of more than a single amino acid; ↑, cleavage site on sugar chain (see Fig. 12.4.2).

Add the same reagents to the control, substituting sodium acetate for enzyme.

A 5- to 10-fold excess of nonionic detergent is required to stabilize the enzyme. As in the previous protocol the amount of sample used as control versus digest depends on the experiment.

3. Incubate the mixture overnight at 30° to 37°C.

Some inactivation of the enzyme occurs at 37°C, even with nonionic detergent present; however, the enzyme is present in sufficient excess that incubation can generally be carried out successfully at this temperature.

4. Inactivate Endo F_2 by heating 5 min at 100°C.

5. Estimate the number of N-linked oligosaccharide chains on the protein (see Support Protocol 1) and/or recover and analyze these chains (see Support Protocol 2). Store protein sample at –20°C.

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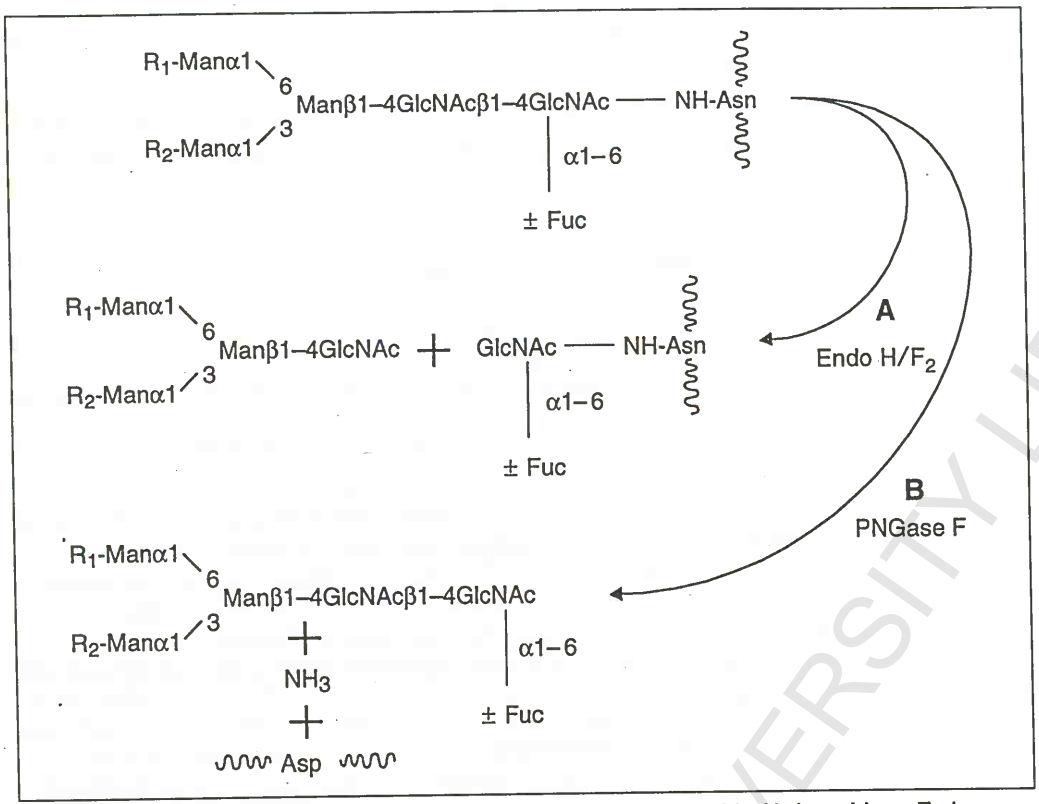

Figure 12.4.2 Comparison of endoglycosidase H or F_2 and peptide:*N*-glycosidase F cleavage reactions. The generic core structure for an N-linked oligosaccharide on a protein is shown at the top. Arrow A indicates products of Endo H or Endo F_2 cleavage and arrow B points to the products of PNGase F cleavage.

PEPTIDE:*N*-GLYCOSIDASE F DIGESTION

BASIC PROTOCOL 3

PNGase F is a glycoamidase that cleaves the bond between the asparagine residue of the protein and the GlcNAc residue that joins the carbohydrate to the protein (Figs. 12.4.1 and 12.4.2). Because it liberates nearly all N-linked oligosaccharides from glycoproteins, it is the preferred enzyme for complete removal of N-linked chains. The glycoprotein sample is denatured and digested with PNGase F to remove N-linked oligosaccharides completely.

Materials

Protein sample
0.1 M 2-mercaptoethanol (2-ME)/0.5% (w/v) SDS (make fresh)
0.5 M Tris·Cl, pH 8.0 (*APPENDIX 2E*)
0.1 M 1,10-phenanthroline in methanol
10% (w/v) nonionic detergent: Triton X-100, Nonidet P-40 (NP-40), 3-(3-cholamidopropyl)-dimethylammonio-1-propane sulfonate (CHAPS), or *n*-octyl-β-D-glucoside
200 to 250 mU/ml PNGase F (Genzyme, Boehringer Mannheim, or Oxford GlycoSystems)

Additional reagents and equipment for estimating the number of N-linked oligosaccharide chains (see Support Protocol 1) or digesting and recovering the chains (see Support Protocol 2)

1. Place ≤10 µl of protein sample containing 75 to 100 µg protein in a microcentrifuge tube. Add 25 µl or 0.1 M 2-ME/0.5% SDS and heat denature 3 to 5 min at 100°C. Cool and microcentrifuge briefly to collect condensed droplets into bottom of tube.

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If more protein is digested, more SDS should be used—at least at a 1.2- to 1.5-fold (w/w) excess over the estimated amount of protein.

2. Place an aliquot of the solubilized, denatured protein sample in a clean microcentrifuge tube for control. To the remaining sample, add the following in order (50 µl total):

   25 µl 0.5 M Tris·Cl, pH 8.0
   10 µl 0.1 M 1,10-phenanthroline
   10 µl 10% nonionic detergent
   5 µl 200 to 250 mU/ml PNGase F.

   Add the same reagents to the control, substituting 0.5 M Tris·Cl, pH 8.0, for enzyme.

   As in the previous protocols, the amount of sample used as control versus digest depends on the experiment.

   Sodium phosphate or HEPES buffer, pH 7.0, can be used instead of Tris·Cl. Phosphate and Tris are most commonly used. Avoid potassium buffers because these may cause precipitation of a potassium-SDS salt. The rare trace amount of metalloproteinase contaminant in some preparations will be inhibited by 1,10-phenanthroline. Use of a nonionic detergent is essential, because SDS inactivates PNGase F. A 5- to 10-fold (w/w) excess of any of the above nonionic detergents over the amount of SDS will stabilize the enzyme.

3. Incubate overnight at 30°C to this temperature.

   Some inactivation of the enzyme occurs at 37°C, even with nonionic detergent present; however, the enzyme is present in sufficient excess that incubation can generally be carried out successfully at 37°C.

4. Inactivate the enzyme by heating 3 to 5 min at 100°C.

5. Estimate the number of N-linked oligosaccharide chains on the protein (see Support Protocol 1) and/or recover and analyze these chains (see Support Protocol 2). Store protein sample at –20°C.

SUPPORT PROTOCOL 1

ESTIMATING THE NUMBER OF N-LINKED OLIGOSACCHARIDE CHAINS ON A GLYCOPROTEIN

One widely used application of these digestions, either alone or in sequence, is to estimate the number of N-linked oligosaccharide chains on a given glycoprotein. This is done by creating a "ladder" of partially digested molecules that differ by only one N-linked sugar chain. The number of separate bands in a one-dimensional polyacrylamide gel (less 1 for the totally deglycosylated protein) provides an estimate of the number of N-linked chains. The conditions used to generate the partially deglycosylated protein must be determined for each protein because the sensitivity of each chain may be different, even when all are completely exposed by denaturation. For this protocol, either the incubation time or the amount of enzyme is varied to determine the conditions required to produce the ladder of partial digests. Usually five or six data points are enough to provide a reasonable estimate (Fig. 12.4.3). Of course, it is important to use enough enzyme to obtain complete deglycosylation so as to ascertain the position of the protein when it lacks any N-linked chains. This is best done by monitoring the effects of PNGase F on newly synthesized [^{35}S]Met pulse-labeled protein just after synthesis but before any N-linked oligosaccharide processing has occurred. Alternatively, a processing inhibitor such as deoxymannojirimycin could be added along with Endo H during the digestion. This protocol can also be used to count the number of high-mannose and complex chains by performing sequential digestions, first with Endo H (specific for high-mannose oligosaccharides) and then with PNGase F (which removes essentially all N-linked chains). The graded

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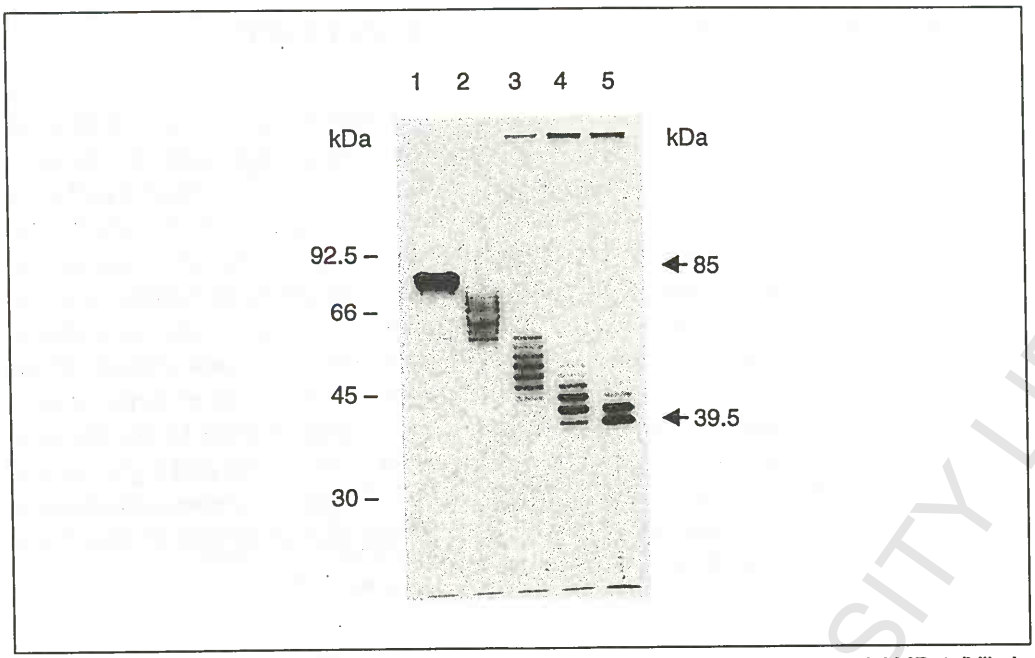

Figure 12.4.3 Data from the estimation of the number of glycosylation sites on LAMP-1 (Viitala et al., 1988). The LAMP-1 (Lysosome-Associated Membrane Protein) glycoprotein contains 18 potential N-linked sites. Graded digestion with increasing amounts of PNGase F was used to generate this ladder of glycoforms: each band contains at least one less N-linked chain than the band above it. An average N-linked carbohydrate chain has an apparent mass of ~1.5 to 3 kDa. Lysosomal membrane glycoprotein was immunoprecipitated from [^{35}S]Met-labeled cells and the sample digested with PNGase F: untreated (lane 1); 5 min (lane 2); 20 min (lane 3); 45 min (lane 4); and 24 hr (lane 5). Figure courtesy of Dr. Minoru Fukuda.

conditions must be established for each digestion. Pulse labeling of protein for 10 min with [^{35}S]Met followed by Endo H digestion is the best way to be sure that all chains are removed. Similarly labeled molecules chased for prolonged times are the best candidates to use for the sequential digestion to estimate the numbers of different types of chains.

1. Add the appropriate 2-ME/SDS solution (see Basic Protocols 1, 2, and 3) to a protein sample and heat denature by incubating 3 to 5 min at 100°C. Aliquot the sample to the number of tubes required to cover the concentration range (e.g., 0.01 to 1 mU/ml) or incubation times (e.g., 5 to 60 min) plus one for the undigested control.
2. Add the appropriate reaction mix for the enzyme being used for digestion (see Basic Protocols 1, 2, and 3).
3. Incubate at 30°C.

   High enzyme concentration (25 mU/ml) and prolonged incubation (16 hr) must be among the conditions included to ensure that there is a data point for maximum deglycosylation.
4. Inactivate the enzyme by heating 5 min at 100°C.
5. Analyze the sample from each concentration/time point, including undigested sample, by one-dimensional SDS-PAGE (*UNIT 10.1*) and detect by autoradiography or immunoblotting.

   Most N-linked chains have a M_r in the range of 1500 to 3000, and loss of one chain is sufficient to change the migration of a protein. This procedure has been used to count up to eighteen N-linked sites on one molecule. A sample result is shown in Figure 12.4.3.

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SUPPORT PROTOCOL 2

DIGESTION AND RECOVERY OF RELEASED N-LINKED OLIGOSACCHARIDE CHAINS

Released oligosaccharide chains can be separated from protein by gel-filtration chromatography, then recovered and analyzed. Assuming that the sugar chains are radiolabeled, they can be detected by scintillation counting; however, if they are not labeled and there is enough material, other chemical methods for detecting sugars should work. Figure 12.4.4 diagrams the steps for this procedure. If the sample contains a mixture of glycoproteins and low-molecular-weight carbohydrate-containing molecules (e.g., as with total cell lysates or membrane preparations), the low-molecular-weight contaminants must be removed by gel-filtration chromatography to prevent contamination of the oligosaccharide chains subsequently released. Once the sample is free of contaminants, it is digested and the protein is separated from released oligosaccharides by gel-filtration chromatography. If the sample is a purified glycoprotein (*UNIT 9.1*), the first eight steps of this protocol can be omitted. The success of the protocol requires complete inactivation (by denaturation) of endogeneous proteases in the sample as well as their absence from the endoglycosidase or glycoamidase preparations.

Materials

Protein sample
0.1% to 1.0% (w/v) SDS
Gel-filtration column (see recipe), calibrated and equilibrated in 0.1% SDS/0.05 M Tris·Cl, pH 7.0
Bovine serum albumin (BSA)
100% acetone, –20°C
0.1 M 2-mercaptoethanol (2-ME)/0.2% (w/v) SDS
0.1% (w/v) SDS/0.05 M Tris·Cl (pH 7.0) *or* HEPES (pH 7.0)
Saturated KCl
Biobeads SM2 (Bio-Rad)
Saturated $AgNO_3$ (store in the dark)

100°C water bath
50-ml conical polypropylene (acetone-resistant) centrifuge tubes
IEC Clinical centrifuge or equivalent
15-ml thick-walled conical glass centrifuge tubes
26-G needle
Bath sonicator (Bransom Ultrasonics) or sonicator with a small tip (3-mm diameter; Kontes Glass)
Vortex Evaporator (Labconco), lyophilizer, or Speedvac evaporator
95°C water bath

Remove low-molecular-weight contaminants

1. Add 0.1% to 1% SDS to the protein sample (keeping the total volume ≤100 µl) and heat 3 to 5 min at 100°C.

   The amount of protein used will depend upon the experiment. The amount of protein dictates how much SDS is needed to solubilize and denature the sample. At least a 1.5× excess over estimated amount of protein should be used.

2. Dilute sample to 0.1% to 0.2% SDS in <0.5 ml and load onto a calibrated gel-filtration column equilibrated in 0.1% SDS/0.05 M Tris·Cl, pH 7.0. Elute with this buffer at the highest usable flow rate. Collect 0.6- to 0.8-ml fractions.

   The same gel-filtration column will be used to isolate the released chains. The protein(s) of interest must elute in the void volume of the column (V_o; see Reagents and Solutions).

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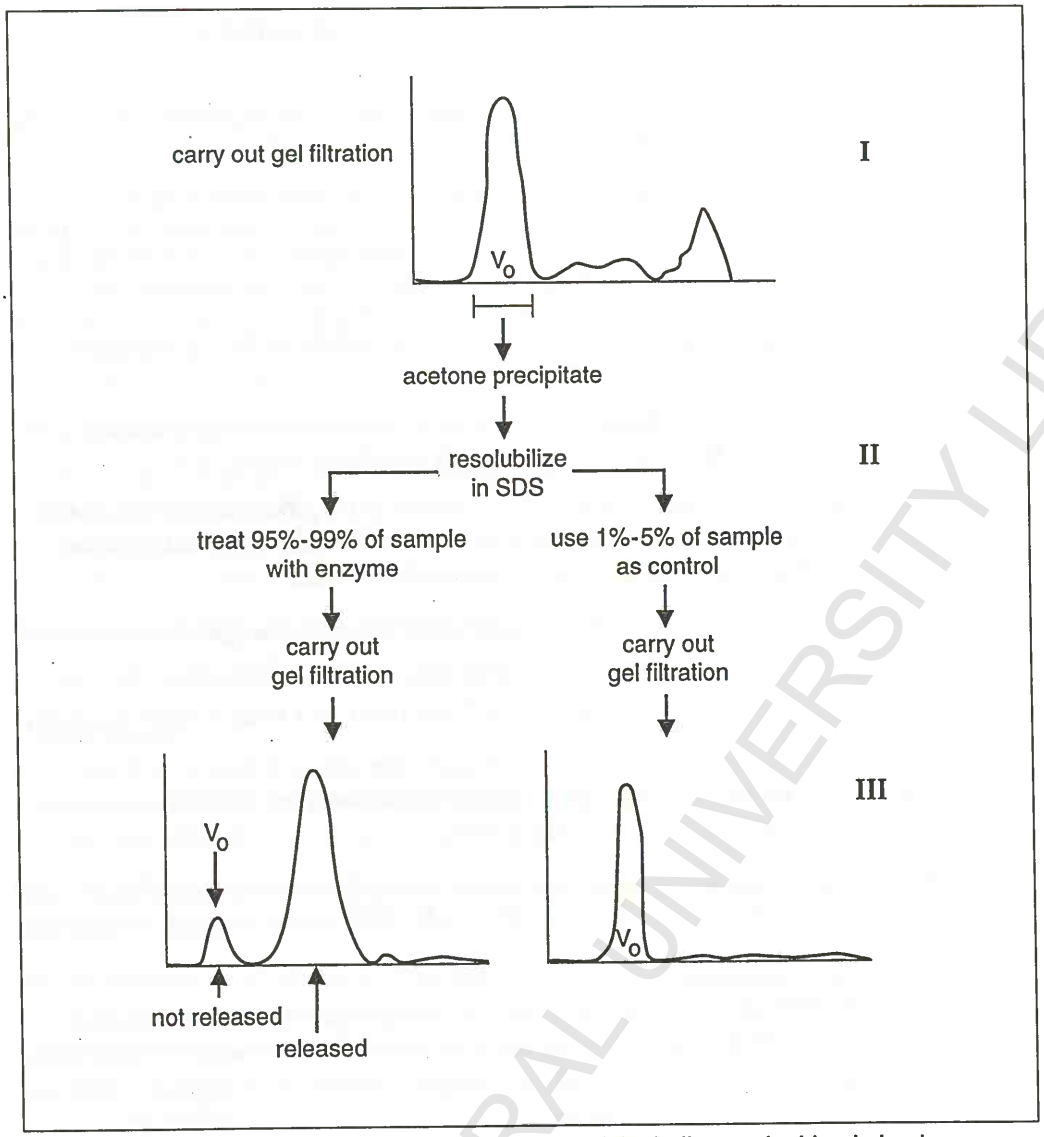

Figure 12.4.4 Flow chart showing the release of N-linked oligosaccharide chains by enzyme digestion and their recovery by gel-filtration chromatography. In part I, gel filtration is used to separate the macromolecules from low-molecular-weight contaminants. In part II, acetone is used to precipitate the proteins, and the proteins are redissolved in SDS. 95% to 99% of the protein is digested with the enzyme of choice. A control of 1% to 5% is incubated in the absence of enzyme. In part III, both samples are analyzed again by gel filtration on the same column as in part II to locate the fractions with the released oligosaccharide chains. Very little should be released from the control. If much "released" material is seen, this indicates that proteolysis of the sample occurred, and the material will consist of glycopeptides, not oligosaccharides. If the control shows little released material, the released fractions are pooled, concentrated, and desalted for further analysis.

3. Pool void volume fractions (~3 to 4 ml). If the total amount of sample glycoprotein is <200 µg, add 100 to 200 µg BSA (as a carrier) to the pooled sample.

   Approximately 200 µg total protein is required to produce a visible precipitate during acetone precipitation.

4. Transfer sample to an acetone-resistant polypropylene 50-ml conical centrifuge tube. Precipitate macromolecules by adding 9 vol of 100% acetone chilled to –20°C. Incubate ≥2 hr at –20°C.

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5. Centrifuge 10 min at 1750 × *g* (top speed in IEC Clinical centrifuge).

A fine, loose pellet should be visible at the bottom of the tube; no or very little haziness should remain in the supernatant. Check the appearance of the supernatant by drawing some of it into a Pasteur pipet.

If the pellet is difficult to see on the bottom of the 50-ml tube, remove all but 2 to 3 ml of the supernatant with a Pasteur pipet, and gently resuspend the material left in the conical tip in the remaining 2 to 3 ml. Transfer this solution to a 15-ml thick-walled conical clear glass tube. Wash the tip and the sides of the polypropylene tube with 1 to 2 ml of 90% acetone chilled to –20°C, and add this to the glass tube. A very fine precipitate should be visible in the glass tube. Centrifuge as above to pellet macromolecules. A 10 to 20 µl pellet is easily visible in this tube.

Do not vortex either tube because vortexing may distribute the small amount of pellet all over the sides of the large tube, resulting in significant sample loss.

6. Remove most of the supernatant with a Pasteur pipet. Wearing gloves, twist a Kimwipe to make a wick and carefully touch it to the side of the tube, below the meniscus but above the pellet, to absorb most of the solvent.

Do not completely air dry the pellet because it will become very difficult to resuspend.

7. Suspend the pellet in 0.1 to 0.2 ml water by sonication (*do not vortex*) for several minutes in a bath sonicator or with a 1- to 2-sec burst of a small (3-mm-diameter) sonicator probe.

8. Solubilize the pellet by adding 0.1 M 2-ME/0.2% SDS, keeping the total volume <0.3 ml. Incubate 10 min at 37°C and 5 min at 95°C.

This incubation will evaporate the acetone and gradually solubilize the pellet. A larger amount of material will require more water and/or SDS to be completely solubilized.

Carry out enzymatic digestion

9. Place 95% to 99% of the sample in a microcentrifuge tube and digest with Endo H (see Basic Protocol 1), Endo F_2 (see Basic Protocol 2), or PNGase F (see Basic Protocol 3). Place the remainder of the sample (control) in a separate tube and incubate in the same way but without enzyme.

10. After digestion is complete, dilute sample to 0.3 ml with 0.1% SDS/0.05 M Tris·Cl (pH 7.0) or HEPES (pH 7.0).

Separate released oligosaccharides

11. Apply sample to the same calibrated gel-filtration chromatography column used in step 2. Elute the column with 0.1% SDS/0.05 M Tris·Cl, pH 7.0. Collect 0.5-ml fractions, using the highest flow rate compatible with the column.

12. Count aliquots of each fraction to detect released oligosaccharide chains in the column fractions. Compare the elution profiles of digested and control samples (Fig. 12.4.2) and determine which fractions contain an amount of released oligosaccharide chains at least 10-fold greater than the amount found in the same region for the control. Pool those fractions into a thick-walled 12-ml conical glass tube.

For nonradiolabeled samples, an appropriate colorimetric assay (UNIT 3.4) can be used if enough material is available.

Most released oligosaccharide chains will elute after the V_o on Sephadex G-50. Only very large or highly charged chains will elute within or near the V_o. If no apparent release is detected, the released sugar may be eluting in the V_o. To check for this possibility, run control and digested samples on a calibrated Sephacryl S-200 column

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in the same buffer. Released oligosaccharide chains that run in the V_o of a Sephadex G-50 column will be retained within a Sephacryl S-200 column of similar dimensions.

There should be very little released material outside of the V_o in the control. If broadening of the peak, trailing, or smearing throughout the column are observed, enzymatic breakdown of the sample (e.g., proteolysis) may have occurred; however, these are very uncommon under these digestion conditions.

13. Remove SDS from the pooled fractions by adding 0.01 vol saturated KCl. Incubate ≥2 hr on ice to maximize SDS precipitation (oligosaccharides will remain in solution).

14. Centrifuge pooled oligosaccharides 5 min at 1750 × *g* to remove the precipitated potassium-SDS salt.

    The conical glass tube must be thick-walled to withstand the centrifugation.

15. Remove unwanted residual nonionic detergent from the Endo F_2 or PNGase F digestions by passing pooled oligosaccharides slowly over a 1-ml Biobead SM2 column connected to a 26-G needle to restrict the flow rate. Wash with five column volumes of water. Pool wash fractions that contain oligosaccharides.

16. Lyophilize the pooled wash fractions or dry on a Vortex-Evaporator. Count a small aliquot of the pool to monitor recovery, which should be essentially complete.

Desalt sample

17. Dissolve lyophilized oligosaccharide sample in 0.3 to 0.5 ml ice-cold water in a microcentrifuge tube. Microcentrifuge 2 min at maximum speed to remove any residual potassium-SDS salt.

18. Desalt sample by running on a 0.5 × 40–cm column of Sephadex G-25, eluting with water. Collect 1-ml fractions.

19. Monitor or assay aliquots of the fractions to locate oligosaccharides. Assay for salt (residual KCl from precipitation of SDS) by mixing a 10-µl aliquot of the fraction with 10 µl saturated $AgNO_3$ on a glass slide. Watch for the formation of a precipitate ($AgCl_2$); this indicates that salt is present. Pool oligosaccharide-containing fractions that are salt-free.

    It is important that the oligosaccharides be salt-free because subsequent treatments, such as glycosidase digestions, are usually conducted in very small volumes (<50 µl) where a small amount of residual salt from the pooled fractions is concentrated many times. Most oligosaccharides will elute separately from salt; however, very small oligosaccharides may elute with salt. At this point, any residual SDS should be below its critical micelle concentration (CMC) and will not run in the V_o with the oligosaccharides.

20. Lyophilize the pooled oligosaccharides or dry them on a Vortex-Evaporator or Speedvac evaporator. Dissolve the free oligosaccharides in water and freeze at –20°C (they will be stable indefinitely if free of salt and other contaminants).

    The final dried or lyophilized material should appear completely dry. If it appears viscous or oily, it may contain residual detergent, which can be removed by passing the material over a 1-ml column of Biobeads SM2 as described in step 15. When purifying small amounts of oligosaccharide (e.g., radiolabeled), large-volume pools from Sephadex G-50 or Sephacryl S-200 columns can contribute nonionic detergent to the mixture. Detergent may interfere with subsequent treatments of the oligosaccharides, such as HPLC analysis or exoglycosidase digestions.

    A modification of this procedure can be used to sequentially isolate sugar chains cleaved by each of the enzymes. First, sensitive chains are cleaved with Endo H and the released high-mannose and hybrid chains collected by gel-filtration chromatog-

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raphy. Protein-bound material is then reisolated from the void volume fraction by acetone precipitation, the acetone is removed, and the protein is redissolved in SDS. The digestion is repeated using Endo F_2 to release biantennary chains, followed by isolation, precipitation, recovery, and dissolution. Finally, the sample is digested with PNGase F and the released tri- and tetraantennary chains isolated by gel-filtration chromatography.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Gel-filtration columns

Hydrate Sephadex G-25 and Sephadex G-50 (Pharmacia Biotech) according to supplier's instructions (Sephacryl S-200 is supplied hydrated). Pack the bottom of the column with a small plug of glass wool over the frit to prevent clogging. Pour a ~0.5 × 40–cm column using Sephadex G-25, and a ~0.5 × 50–cm column using either Sephadex G-50 or Sephacryl S-200. Calibrate the column using dextran blue to mark the void volume and glucose to mark the fully included volume and usable range of the column. Detect glucose using the phenol–sulfuric acid test (Manzi, 1993).

Alternatively, any tritiated monosaccharide may be used instead of glucose (see Critical Parameters) and detected by liquid scintillation counting. Columns may be reused many times.

COMMENTARY

Background Information

Endoglycosidase H

Endoglycosidase H (Endo H) cleaves the bond between the two GlcNAc residues in the core of N-linked oligosaccharides. One GlcNAc residue remains attached to the protein or peptide and the remainder of the chain is released as an intact unit (Fig. 12.4.2; Tarentino et al., 1989). The oligosaccharide structures cleaved by Endo H are shown in Figure 12.4.1. and schematically in Figure 12.4.5. Substrates for Endo H include all high-mannose oligosaccharides and certain hybrid types but not bi-, tri-, tetra-, or pentaantennary (complex-type) chains. Endo H also cleaves oligosaccharides that have α1-6 fucose residues bound to the reducing (protein to carbohydrate linkage) GlcNAc residue. The enzyme acts on reduced and nonreduced free oligosaccharides (e.g., those released by PNGase F cleavage) by removing the terminal GlcNAc(itol) from a reduced sugar chain that has an intact chitobiose core. It will cleave sugar chains from intact glycoproteins, glycopeptides, or glycopeptides with only asparagine (Tarentino et al., 1989).

The specificity of Endo H has been extensively exploited to trace the movement of newly synthesized glycoproteins through the endoplasmic reticulum (ER) and Golgi apparatus (Rothman and Orci, 1990; Mellman and Simons, 1992). Proteins remain sensitive to Endo H while they are in the ER and in early cis portions of the Golgi; they become Endo H–resistant after they are processed by enzymes located in the middle (medial) Golgi. Figure 12.4.5 shows the normal pathway for N-linked oligosaccharide maturation. Endo H cleaves N-linked oligosaccharides from proteins as long as they have not lost one of the mannose residues cleaved by Golgi mannosidase II. After removal of a mannose the oligosaccharide becomes resistant to Endo H cleavage. Addition of the first GlcNAc residue, removal of the two additional Man residues, and addition of the second GlcNAc residue are thought to occur very rapidly and within a single compartment in the medial Golgi. [Another view of the relationship between glycoprotein processing and the structure of the Golgi, however, holds that the boundaries between these compartments are less clear-cut (Mellman and Simons, 1992).] Fortunately, sensitivity and resistance to Endo H are independent of the evolving concepts of Golgi structure; they provide mileposts for an incomplete map of subcellular trafficking. In practice this is usually determined by pulse labeling newly synthesized proteins with ^{35}S-labeled amino acids, followed by analysis by one- or

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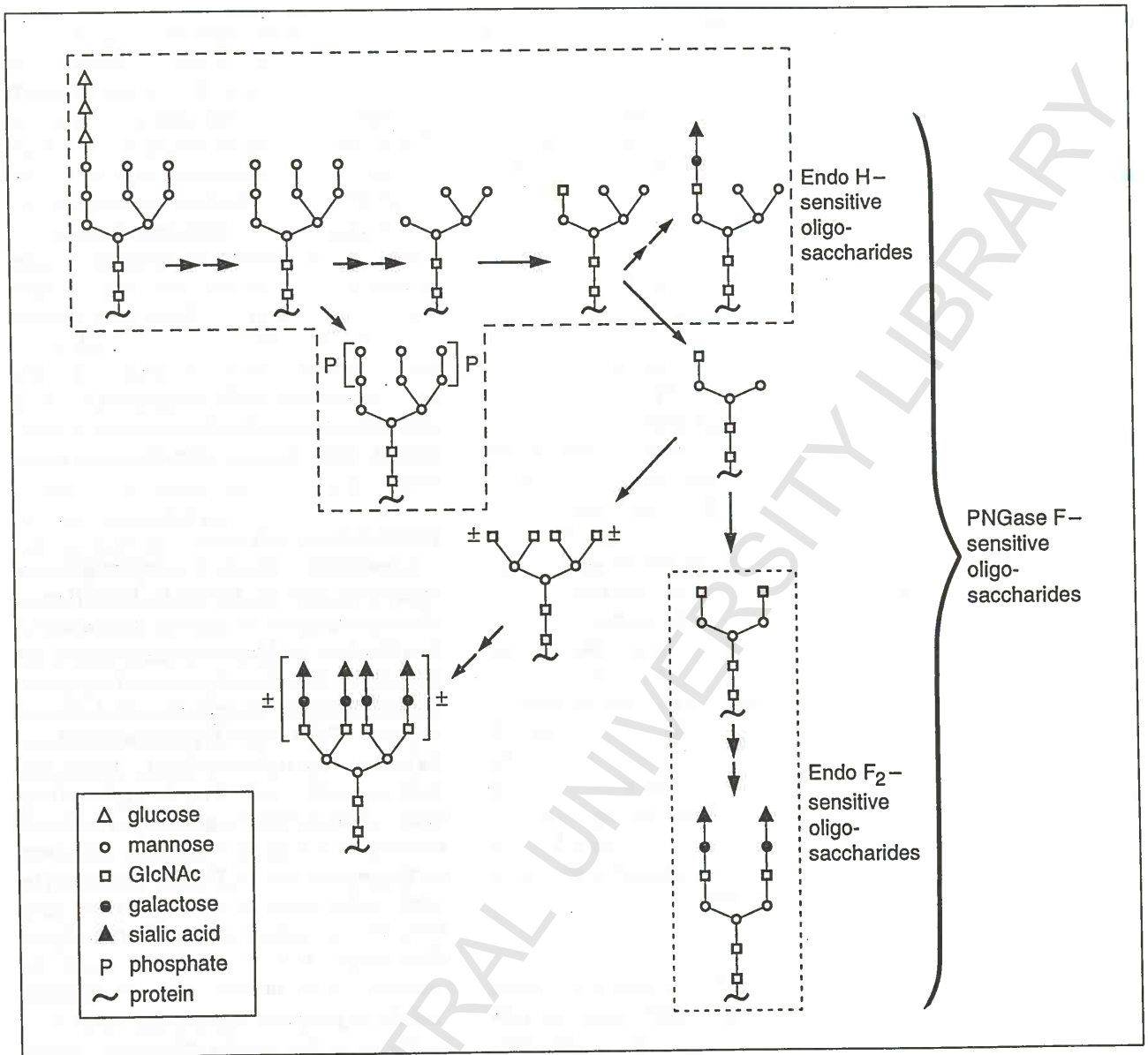

Figure 12.4.5 The N-linked oligosaccharide processing pathway and enzymes used for cleavage. The symbol ± indicates the oligosaccharide is sensitive whether or not residue is present.

two-dimensional gel electrophoresis (*UNITS 10.1-10.4*) and autoradiography to detect mobility changes.

Endoglycosidase F and Peptide N-glycosidase F from Flavobacterium meningosepticum

Elder and Alexander (1982; Alexander and Elder, 1989) made a landmark discovery when they identified an enzyme in culture filtrates of the bacterium *Flavobacterium meningosepticum* that cleaved N-linked oligosaccharides from glycoproteins. This preparation had a broad substrate specificity. The endoglycosidase activity (Endo F) in this preparation is actually due to a set of enzymes with a more restricted substrate range. Like Endo H, these enzymes cleave the sugar chains between the two core GlcNAc residues (Fig. 12.4.2). Until recently, Endo F was thought to be a single enzyme, but it is now clear that Endo F preparations contain at least three different enzymes, each with a distinct specificity (Plummer and Tarentino, 1991). Endo F_1 is very similar in specificity to Endo H, while Endo F_2 prefers biantennary chains and Endo F_3 will cleave bi- and triantennary but not tetraantennary chains (Fig. 12.4.5). These enzymes should prove very useful for differentiating between various types of N-linked chains. However, only Endo F_2 is currently commercially available (from Genzyme).

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Subsequently, Plummer et al. (1984) carefully analyzed the *F. meningosepticum* filtrates and resolved at least two distinct activities. One, a glycoamidase, accounts for the very broad substrate range: it releases the *entire* carbohydrate chain from the protein by cleaving the asparagine-GlcNAc bond (Fig. 12.4.2). The enzyme is called by various names, including peptide:*N*-glycosidase F (PNGase F), glycopeptidase F, and *N*-glycanase (Genzyme), but the proper name is peptide-*N*-4(*N*-acetyl-β-glucosaminyl)asparagine amidase F. PNGase F digestion releases sensitive oligosaccharide chains from proteins and from tryptic or chymotryptic peptides, but not from proteins digested with proteases such as pronase or proteinase K, because both the carboxyl and amino groups of the asparagine residue must be in peptide linkages in order for PNGase F to attack. The enzyme will not cleave oligosaccharides that contain fucose linked α1-3 to the GlcNAc bound to asparagine of the peptide (Tretter et al., 1991). This structure is very common in plant glycoproteins, and has been found in one insect, but has not yet been observed in higher animals. If digestion with PNGase F fails to produce a change in the molecular weight of a protein, or to release radiolabeled sugar chains, this may indicate that the protein contains this type of fucose residue, or another as yet undefined substituent that prevents cleavage.

Other enzymes

Endoglycosidase D (Endo D) and glycopeptidase A are also commercially available (ICN Biomedicals). Endo D has a very limited specificity and is not often used. Glycopeptidase A does not work well on large intact glycoproteins, but will cleave many types of oligosaccharide chains from tryptic glycopeptides, including plant glycopeptides with fucose linked α1-3 to the innermost core GlcNAc. It will also release the GlcNAc that remains on peptides following Endo H or F_2 cleavages. Their limited substrate ranges and high price make these enzymes less attractive than the others described in detail here.

Deciding whether to buy or purify these enzymes

This decision will depend on whether the enzyme(s) will be used frequently or for just a few digestions (e.g., 10 to 20). If the latter, then it is probably best to buy the enzyme(s) as needed. If they will be used often, in-house purification should be considered. The procedure described by Tarentino and Plummer (1994) provides nearly pure PNGase F and Endo F_1 in a single step; it begins with 12 liters of culture medium and ends with 5 mg of PNGase F and Endo F_1 and <0.5 mg of Endo F_2 and Endo F_3. At current prices, this amounts to ~$1,000,000 worth of commercial PNGase alone! The digestions using these in-house-purified enzymes should be performed as described in the basic protocols with a metalloprotease inhibitor; this should give satisfactory results. The complete substrate specificities of Endo F_2 and Endo F_3 are still being worked out, so they are not useful today for diagnosing oligosaccharide structure. However, both enzymes are stable for at least 1 year at 4°C.

Critical Parameters

Commercial "Endo F" preparations are mixtures of Endo F_1, F_2, and F_3. Endo F preparations should not be used for routine deglycosylation or to draw conclusions about the structure of the released oligosaccharides unless the specificity is clearly defined. As shown in Figure 12.4.5, Endo H releases most high-mannose and hybrid chains (Varki and Kornfeld, 1983) and Endo F_2 preferentially (20:1) releases biantennary chains. PNGase F releases all of these as well as tri-, tetra-, and pentaantennary chains. These enzymes can be used in the order Endo H, Endo F_2, then PNGase F to release oligosaccharide chains differentially; PNGase is the best choice for complete release of intact N-linked oligosaccharides from denatured proteins.

For all of the enzyme digestions, sample denaturation is the most critical step in the procedure. Degradative enzymes such as glycosidases and proteases may be present in crude samples and may contribute to variable and unwanted degradation of protein or carbohydrate chains. Denaturation with SDS inactivates these enzymes and prevents confusion about the enzyme responsible for cleavage. Maximum deglycosylation of glycoproteins using any of the enzymes is achieved only when the glycoproteins are completely denatured. SDS is the best denaturant, but a 5- to 10-fold weight excess of nonionic detergent to protein must be added to prevent inactivation of Endo F_2 and PNGase F by SDS. The choice of nonionic detergent depends upon the method used to analyze the digested sample. *n*-octyl-β-D-glucoside and CHAPS are dialyzable, whereas NP-40 and Triton X-100 are not. Each can be removed by slowly passing the digest over an

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~1-ml column of Biobeads SM2. Alternatively, it is possible to use 2.5 M urea at 37°C to denature the glycoprotein; the enzyme will retain partial activity for 24 hr in urea. Samples that have been precipitated with acetone should be heated long enough to evaporate the acetone before or during solubilization with SDS.

The amount of enzyme recommended in the protocols is in large excess and should be sufficient to cleave the majority of sensitive oligosaccharide chains.

Endo H

Endo H has a broad pH optimum between 5.5 and 6.5, and phosphate or citrate/phosphate buffers can be used in place of citrate. Endo H is very stable to proteases, freezing and thawing, and prolonged incubations. No additives are required for storage of the enzyme. At concentrations below 5 to 10 µg/ml (200 to 400 mU/ml), Endo H will bind to glass, so it should be stored in plastic vials (e.g., screw-cap microcentrifuge tubes). It is advisable to avoid using glycerol in the incubation mixtures, because Endo H will transfer glycerol to the free reducing end of the newly liberated sugar chain. This is especially critical if the cleaved oligosaccharide will later be labeled at the reducing terminus with sodium borotritide (Fukuda, 1994). PMSF in the reaction mixture will inactivate trace serine protease that may accompany some preparations of natural Endo H and which could lead to erroneous results.

Endo F_2

Endo F_2 has a broad pH optimum of 4.0 to 6.0 and retains >50% of its activity at pH 7.0. Like PNGase F, the enzyme is sensitive to SDS alone, but adding nonionic detergents prevents denaturation of the enzyme by SDS. Genzyme supplies Endo F_2 in 10 mM sodium acetate buffer, pH 4.5, on dry ice. The enzyme is stable for months at 4°C; it can also be frozen in screw-cap microcentrifuge tubes in aliquots at –70°C, but repeated freeze/thaw cycles should be avoided. 1,10-phenanthroline is used to inhibit a zinc metalloprotease that may be a trace contaminant of the enzyme preparation. Endo F_2 has only recently become commercially available and its activity has not been very thoroughly evaluated, so it is advisable to interpret results cautiously. This enzyme is used to obtain evidence for the presence of biantennary oligosaccharide chains. It is relatively expensive compared to the other enzymes, and its preference for biantennary chains over high-mannose type chains is about 20-fold, so Endo H and PNGase F are better choices for broadly distinguishing high-mannose from complex-type chains.

PNGase F

PNGase F has a broad pH optimum (pH 6.5 to 9.0), but is best used around pH 7.0 to avoid losing labile sugar substituents on sialic acids, such as *O*-acetyl esters (Roux et al., 1988). Commercial PNGase F is Endo F–free and is stable for 6 months at 4°C or indefinitely at –70°C. However, it should be stored in small aliquots and repeated freeze/thaw cycles should be avoided. PNGase F will bind to glass and plastic surfaces and should not be stored in dilute solutions of <0.1 mU/ml. All of the unit activities of commercial preparations are based on cleavage of dansylated glycopeptides; they are expressed in nmol/min, which are actually mU, not true International Units (1 IU = 1 µmol/min). SDS inactivates PNGase F, but adding a 5- to 10-fold weight excess of nonionic detergent protects the enzyme.

Gel-filtration matrix

The choice of gel-filtration matrix to use in separating cleaved oligosaccharide chains from the parent protein depends upon the size of the protein and the apparent size of the released oligosaccharide chains. The critical point is that, once released, the oligosaccharides must be separable from the deglycosylated protein—the oligosaccharide should elute in the included volume and the protein in the V_o. Very large or highly anionic oligosaccharide chains released by the digestion may still elute in the void volume of a Sephadex G-50 column (see *UNIT 12.1*), which excludes proteins larger than 50 kD. These released chains would thus *appear* to be still bound to protein (Roux et al., 1988). However, even these less common types of sugar chains are separated from proteins on Sephacryl S-200 which excludes proteins larger than 200 kD (see Table 8.3.2). On the other hand, a small glycoprotein that elutes in the void volume of a Sephadex G-50 column may not elute in the void volume of a Sephacryl S-200 column and once the oligosaccharides are released, they may not separate completely from the protein. Usually either matrix is satisfactory for separating most released oligosaccharides from most glycoproteins. Because many proteins have molecular weights between 50 and 200 kD, Sephacryl S-200 is generally the better choice.

Anticipated Results

The majority of known N-linked oligosaccharides will be removed by at least one of the enzymes. However, failure of an enzyme to produce the expected deglycosylation is not proof that the protein does not contain N-linked oligosaccharides. As discussed above, a fucose residue linked α1-3 to the first GlcNAc residue of an oligosaccharide makes it completely resistant to PNGase F (Tretter et al., 1991). Although this structure has only been reported in plants and in one insect, it may be present, but as yet undetected, in higher animals. The presence of sulfate esters in the core region may also cause partial enzyme resistance.

Time Considerations

Digestion can be completed during 1 day or carried out overnight for convenience. Isolation of released radiolabeled oligosaccharide chains and sample desalting take 1 day. The rate-limiting step in the recovery of released oligosaccharides is lyophilization of the material from the Sephadex G-50 or Sephacryl S-200 columns. A Vortex-Evaporator (Labconco) can be a great time-saver for this step, but this equipment is not common.

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Contributed by Hudson H. Freeze
La Jolla Cancer Research Foundation
La Jolla, California

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Detection of Glycophospholipid Anchors on Proteins

Many eukaryotic proteins are tethered to the plasma membrane by glycosyl phosphatidylinositol (GPI) membrane anchors. Figure 12.5.1 schematically depicts the minimal (core) structure common to all GPI protein anchors characterized to date. Detailed structural information is described in reviews by Englund (1993) and McConville and Ferguson (1993).

This unit provides a general approach for detecting GPI-anchored proteins. First, the detergent-partitioning behavior of a protein of interest is examined for characteristics of GPI-linked species. The partitioning of total cellular and isolated proteins with Triton X-114 is described in Basic Protocol 1 and Alternate Protocol 1, respectively. Precondensation of Triton X-114, which is necessary to remove hydrophilic contaminants before partitioning, is outlined in Support Protocol 1.

The protein may also be subjected to specific enzymatic or chemical cleavages to release it from its GPI anchor. Phospholipase cleavage (starting with intact cells or membranes, or with isolated protein, respectively) is detailed in Basic Protocol 2 and Alternate Protocol 2, and chemical cleavage with nitrous acid is described in Basic Protocol 3.

If GPI-anchored proteins are radiolabeled with fatty acids, it facilitates the detection of the GPI protein products following the cleavage reactions. Separation of lipid moieties released from proteins is described in Support Protocol 3 and base hydrolysis of proteins is presented in Basic Protocol 4. Figure 12.5.2 outlines the relationships between the various protocols.

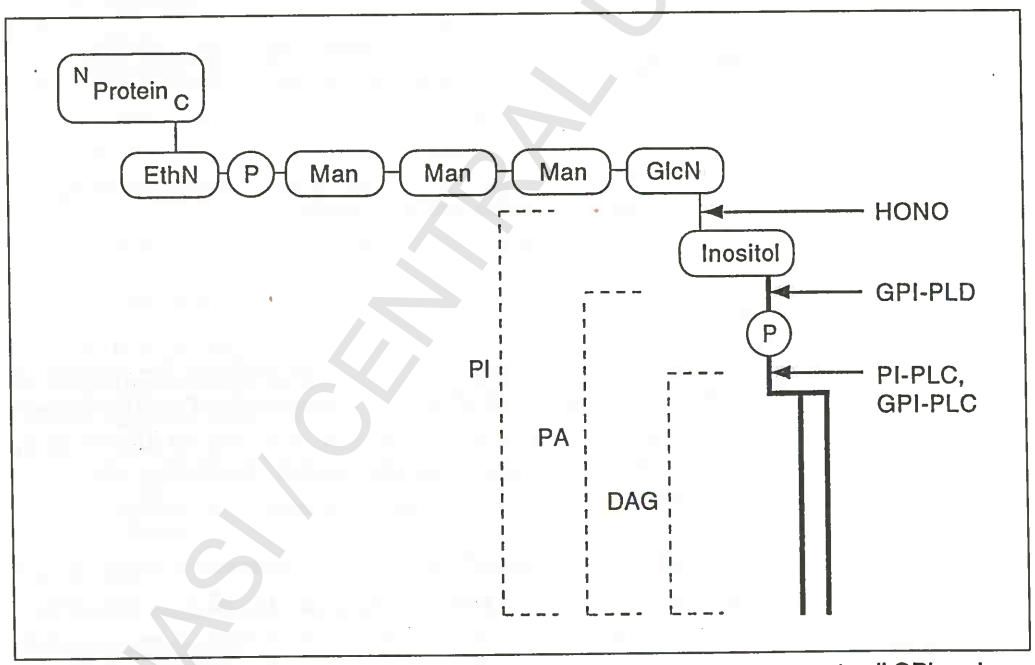

Figure 12.5.1 Schematic representation of the glycan core structure common to all GPI anchors. The sites of cleavage of phospholipase C enzymes (PI-PLC, GPI-PLC), phospholipase D (GPI-PLD), and nitrous acid (HONO) are indicated, as are lipid products resulting from these cleavages: PI (phosphatidylinositol), PA (phosphatidic acid), and DAG (diacylglycerol). Other abbreviations: Man, mannose; GlcN, glucosamine; EthN, ethanolamine; P, phosphate group. Phospholipase C treatment generates inositol cyclic phosphate, the major epitope contributing to the cross-reacting determinant (CRD). Lipid products may vary from those depicted, depending on features of the anchor (see Background Information).

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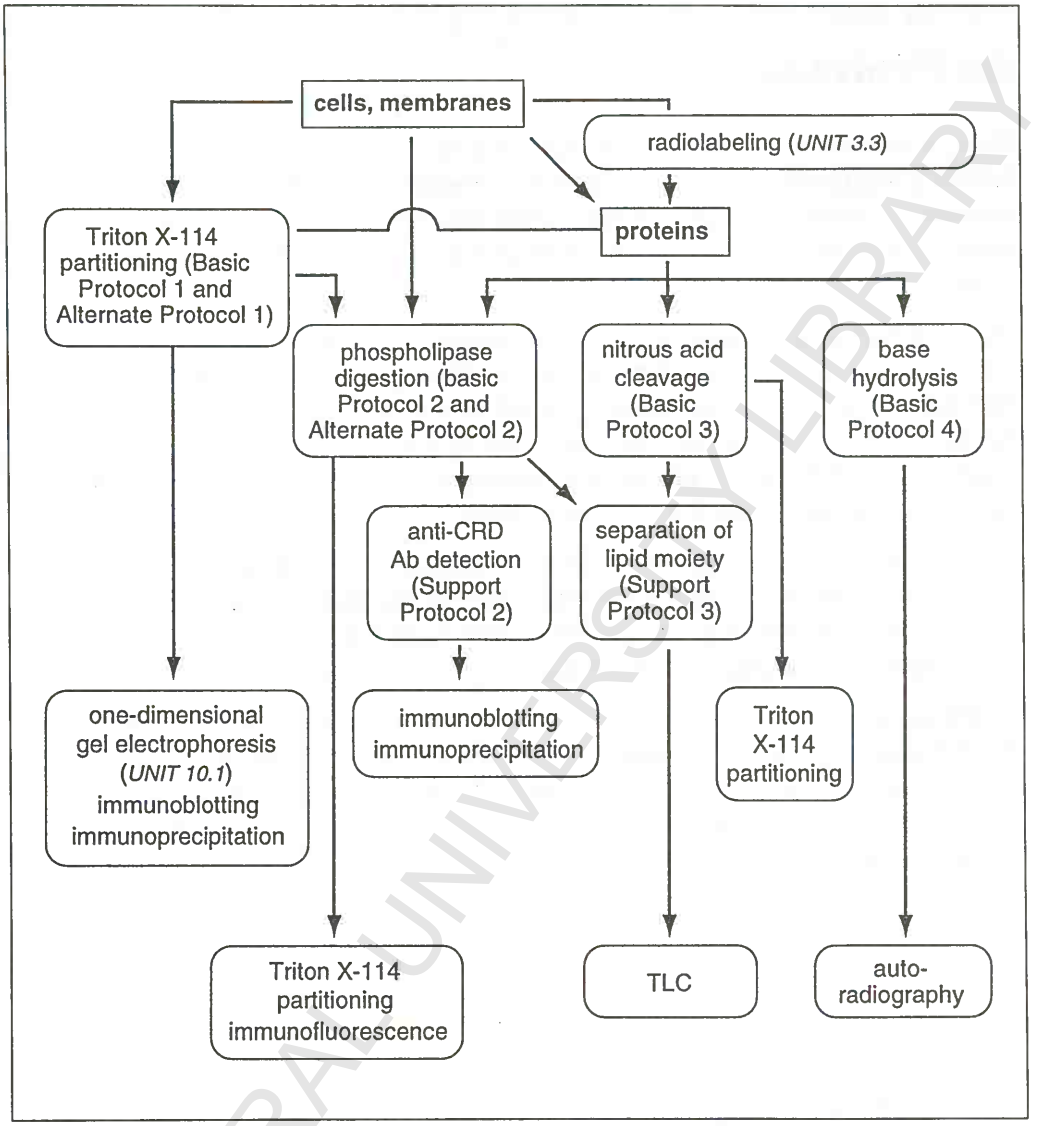

Figure 12.5.2 Flow chart for the detection of GPI-anchored proteins.

STRATEGIC PLANNING

The techniques described below require that a method for identifying the protein of interest be available. Proteins may be visualized after one-dimensional gel electrophoresis (*UNIT 10.1*) and Coomassie blue or silver staining (*UNIT 10.5*), or they may be identified by immunoprecipitation or immunoblotting using available specific antibodies. Enzymes may also be detected by their known activities (e.g., see Kodukula et al., 1992).

It is convenient, particularly for low-abundance species, to use proteins that are labeled with radioactive amino acids (*UNIT 3.3*) or GPI components (see Fig. 12.5.1). The ability to radiolabel proteins with GPI precursors such as fatty acids, ethanolamine, *myo*-inositol, and certain monosaccharide precursors (*UNIT 12.2*) is in itself suggestive of GPI anchorage. Because GPI-anchored proteins commonly represent a small fraction of eukaryotic glycoconjugates, metabolic radiolabeling may be inefficient. Tunicamycin may be used to increase relative incorporation of monosaccharide precursors into GPIs.

Rigorous identification of specific GPI anchor structures requires detailed analysis. These methods, which are beyond the scope of this unit, are described in Ferguson (1992).

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EXTRACTION AND PARTITIONING OF TOTAL PROTEINS FROM CELLS OR MEMBRANES WITH TRITON X-114

BASIC PROTOCOL 1

Extraction of cells or tissues with the detergent Triton X-114 provides a profile (and potentially an initial purification) of amphiphilic proteins, including integral membrane proteins and species bearing GPI anchors. Once a GPI-containing protein is released from the lipid component of its anchor, it will no longer partition into the detergent-enriched phase. This alteration of partitioning behavior provides a rapid assay for the presence or absence of anchors. It may be used to monitor the changes in proteins produced by the other procedures.

In this protocol, cells or membrane fractions are first resuspended in TBS, then incubated on ice with Triton X-114. The mixture is then warmed and separates into two protein-containing phases due to aggregation of detergent micelles (see Background Information). The upper (detergent-depleted) phase contains hydrophilic proteins; amphiphilic proteins (including those with GPI anchors) partition to the lower (detergent-enriched) phase. Both phases are then analyzed to determine the group to which each protein belongs.

Materials

Cells, membrane fraction, or other source of protein
Tris-buffered saline (TBS; see recipe), ice-cold
Precondensed Triton X-114 stock solution in TBS (see Support Protocol 1), ice-cold

15-ml polypropylene centrifuge tubes
Centrifuges: low-speed (tabletop) and equipped with appropriate rotor (e.g., SS-34), at 4°C and room temperature

1. Resuspend cells (or membranes) in ice-cold TBS to a final protein concentration of ≤4 mg/ml in a 15-ml tube.

   The amount of protein required is that which will give detectable levels at the end of the procedure, which will depend on the system the individual researcher is using. As a guideline, use several times the amount of protein that is believed to be needed, to account for partitioning and losses (see Critical Parameters).

2. Add ⅕ vol precondensed Triton X-114 stock solution (~2% final concentration).

3. Extract cells by incubating 15 min on ice with occasional mixing.

   At this point in the procedure there will only be one phase.

4. Centrifuge cell mixture 10 min at 10,000 × *g* (9000 rpm in an SS-34 rotor), 4°C, and transfer supernatant to a fresh tube. Resuspend pellet in ice-cold TBS and save on ice for analysis in step 7.

   The cold supernatant fraction contains both soluble and detergent-extracted proteins; the latter group includes proteins anchored by GPI structures or by transmembrane polypeptide domains. The pellet contains additional GPI species, as well as cell elements insoluble in nonionic detergents.

5. Warm supernatant fraction to 37°C in a water bath; the solution will become cloudy as it reaches 37°C.

6. Centrifuge the solution in a tabletop centrifuge 10 min at 1000 × *g*, room temperature. Collect upper and lower phases in separate tubes.

   The upper (detergent-depleted) phase contains soluble proteins; the lower (detergent-enriched) phase contains proteins anchored by GPI structures or by transmembrane domains.

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7. Analyze the resuspended pellet from step 4 and each phase from step 6 for the proteins of interest.

   Analysis may be by an activity assay, one-dimensional gel electrophoresis and staining of proteins (UNITS 10.1 & 10.5), or immunoprecipitation or immunoblotting using specific antibodies (see Strategic Planning for discussion).

ALTERNATE PROTOCOL 1

PARTITIONING OF ISOLATED PROTEINS WITH TRITON X-114

A modification to the basic protocol for Triton X-114 extraction of proteins from whole cells or membranes is used to partition isolated proteins (see Basic Protocol 1).

1. Dilute or dissolve the protein in 1 ml ice-cold TBS in a 1.5-ml microcentrifuge tube. Add 0.2 ml Triton X-114 and mix.

   See Critical Parameters for guidelines on the total amount of protein to use.

2. Warm protein mixture several minutes (or until it becomes cloudy) in a 37°C water bath. Microcentrifuge 5 min at maximum speed.

3. Collect the upper and lower phases and analyze each phase for the protein(s) of interest.

SUPPORT PROTOCOL 1

PRECONDENSATION OF TRITON X-114 DETERGENT

Triton X-114 often contains hydrophilic contaminants, and must be precondensed by several rounds of phase separation before use.

Materials

Triton X-114 detergent
Tris-buffered saline (TBS; see recipe)

50-ml centrifuge tubes
Tabletop centrifuge

1. Dissolve 1.5 g Triton X-114 in 50 ml TBS, place in a 50-ml centrifuge tube, then chill on ice.

   At this point, the solution will be clear.

2. Warm the solution to 37°C in a water bath.

   Once the solution has warmed to the bath temperature, it will become turbid.

3. Centrifuge the solution in a tabletop centrifuge 10 min at 1000 × *g*, room temperature.

4. Remove and discard the upper (detergent-depleted) phase and redissolve the lower (detergent-enriched) phase in an equal volume of ice-cold TBS.

   Hydrophilic contaminants partition into the upper phase.

5. Repeat partitioning (steps 2 to 4) three times.

   The final detergent-enriched phase contains ~12% detergent. It may be stored at 4°C for routine use or frozen for long-term storage.

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IDENTIFICATION OF GPI-ANCHORED PROTEINS BY PI-PLC DIGESTION OF INTACT CELLS

BASIC PROTOCOL 2

Phospholipase C (PLC) cleaves within the GPI membrane anchors of glycoproteins (see Fig. 12.5.1), causing the release of protein from cell membranes. In this protocol, intact cells or cell membranes are digested with phosphatidylinositol-specific phospholipase C (PI-PLC). Following incubation with the enzyme, the cell mixture is centrifuged and the resulting pellet and supernatant are analyzed for the presence of the protein of interest. Proteins released from GPI anchors are found in the supernatant; lack of release may indicate absence of a GPI anchor, modification of the GPI protein, or other factors (see Background Information).

Materials

Cells (or membrane preparation)
Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC; e.g., from *Bacillus thuringiensis*, Oxford GlycoSystems)
Hanks balanced salt solution (HBSS; e.g., Life Technologies), buffered saline, or culture medium
Tabletop centrifuge and appropriate centrifuge tubes

1. Obtain cells (or membranes) of interest in dispersed suspension. Wash duplicate aliquots twice in HBSS, buffered saline, or culture medium, then resuspend in any of these solutions.

   The final protein concentration of the samples (typically 0.1 to 0.5 mg/ml) should be based on supplier specifications for the enzyme preparation used. See Critical Parameters for guidelines on the total amount of protein to use.

2. Add bacterial PI-PLC to one aliquot. Add no enzyme to the second aliquot (control). Incubate tubes 1 hr at 37°C.

   The amount of enzyme should be determined by supplier recommendations.

3. Centrifuge cells in a tabletop centrifuge 5 min at 1000 × *g*. Remove each supernatant to a fresh tube and save for analysis in step 4. Resuspend pellet in a volume of buffered saline equal to the volume of supernatant removed.

4. Assay supernatant and pellet fractions for protein(s) of interest.

   The proteins may be assayed by Triton X-114 detergent partitioning (see Basic Protocol 1), one-dimensional SDS-PAGE (UNIT 10.1) followed by Coomassie blue or silver staining (UNIT 10.5), immunoprecipitation or immunoblotting.

IDENTIFICATION OF GPI ANCHORAGE BY PHOSPHOLIPASE TREATMENT OF ISOLATED PROTEINS

ALTERNATE PROTOCOL 2

Several specific phospholipases that effectively degrade only GPI species are useful for analyzing GPI anchorage of isolated proteins (see Background Information). In this protocol, aliquots of the protein are digested in parallel with the following phospholipases: bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), a GPI-specific phospholipase C (GPI-PLC) from *Trypanosoma brucei*, and a GPI-specific phospholipase D (GPI-PLD) from mammalian serum (rat, rabbit, or human). Proteins are partitioned by Triton X-114 extraction (see Alternate Protocol 1), then analyzed. Specific cleavage of proteins by phospholipase treatment strongly indicates GPI anchorage.

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Materials

Isolated protein (see Basic Protocol 1)
Acetone, –20°C
Appropriate enzyme buffer: GPI-PLD buffer, GPI-PLC buffer, and PI-PLC buffer (see recipes)
Phospholipase enzymes: GPI-PLD from rat, rabbit, or human whole serum, GPI-PLC from *Trypanosoma brucei* (Oxford GlycoSystems), and PI-PLC from *Bacillus thuringiensis* (Oxford GlycoSystems) or *B. cereus* (Boehringer Mannheim or Sigma)
Tris-buffered saline (TBS; see recipe)
Precondensed Triton X-114 solution (see Support Protocol 1)
Centrifuge and rotor (e.g., SS-34)

1. If the protein sample is lyophilized or concentrated in a buffer similar to the phospholipase buffers, begin at step 3. If it is not, acetone-precipitate the protein by adding 8 vol of –20°C acetone to the protein solution and incubating it ≥3 hr at –20°C.

   See Critical Parameters for guidelines on the total amount of protein to use. For low-abundance material, efficiency of precipitation may be increased by including 15 μg of a carrier protein (e.g., cytochrome c or bovine serum albumin).

2. Centrifuge the sample 20 min at 15,000 × *g* (~11,000 rpm in an SS-34 rotor), 4°C. Aspirate supernatant and allow pellet to air dry.

3. For each enzyme to be tested, dissolve two aliquots of the protein in 100 μl of the appropriate enzyme buffer in 1.5-ml microcentrifuge tubes.

4. To one aliquot from each pair add the appropriate enzyme. Add an equal volume of the appropriate enzyme buffer to the second aliquot (control). Mix tubes and incubate 1 hr at 37°C.

   The amount of enzyme added should be based on the activity of the lipase preparation (see supplier specifications) and the estimated amount of protein present. It may be desirable to titrate the enzyme, using various amounts in parallel reactions, to be sure that all sensitive proteins are completely digested.

   GPI-PLD is not generally available, but whole serum (rat, rabbit, or human) may serve as a source of GPI-PLD activity. Aliquots of serum may be stored frozen and thawed before use. For the assay, use 2 μl whole serum in a 100-μl reaction.

5. Dilute reaction with 200 μl ice-cold TBS, then add 60 μl Triton X-114. Chill 15 min on ice.

6. Partition the proteins (see Alternate Protocol 1, steps 2 and 3).

   GPI-anchored proteins partition into the detergent-enriched phase of Triton X-114 in the control reaction (uncleaved) and into the detergent-depleted phase after cleavage of the GPI anchor by phospholipase.

7. Analyze each phase for the presence of protein(s) of interest (see Basic Protocol 1, step 7).

   Alternatively, PI-PLC and GPI-PLC cleavage products can be detected by reactivity with anti-CRD antibody (see Support Protocol 2). In addition, if the anchor is radiolabeled with fatty acids (UNIT 12.2), cleavage may be assessed by analysis of radiolabeled lipid products.

   Migration of phospholipase-digested proteins on electrophoretic gels is not predictable and should not be used alone to assess cleavage. Either no shift or a shift in either direction (relative to uncleaved protein migration) may be observed upon GPI anchor removal, depending on the properties of the protein.

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DETECTION OF PRODUCTS AFTER PHOSPHOLIPASE TREATMENT BY REACTIVITY WITH ANTI-CRD ANTIBODY

SUPPORT PROTOCOL 2

Cleavage of GPI structures with phospholipase C reveals a cryptic antigen, termed the cross-reacting determinant (CRD). The major epitope contributing to the CRD is an inositol 1,2-(cyclic) monophosphate formed during phospholipase C cleavage; other structural features may also contribute to the antigenicity. Reactivity of a protein with anti-CRD antibody after solubilization by phospholipase C strongly indicates the presence of a GPI anchor. Anti-CRD antibody (Oxford GlycoSystems) may be used after phospholipase C treatment to immunoprecipitate or immunoblot proteins of interest. It should be noted that phospholipase D cleavage does not create the epitope required for anti-CRD activity (see Fig. 12.5.1).

NITROUS ACID CLEAVAGE OF GPI-ANCHORED PROTEINS

BASIC PROTOCOL 3

An unusual property common to all GPI structures is the presence of a nonacetylated glucosamine that is glycosidically linked to inositol. The glycosidic bond is specifically cleaved when the glucosamine undergoes nitrous acid deamination, thereby cleaving the GPI anchor (see Fig. 12.5.1). Because nonacetylated glucosamine rarely occurs outside of GPIs, nitrous acid deamination may be used to identify GPI linkage to protein. Due to side reactions, deamination is not always quantitative or precisely reproducible, but it does serve a useful diagnostic purpose.

In this protocol, the protein is incubated in nitrous acid to cleave the GPI anchors. Cleaved and uncleaved proteins are then separated by Triton X-114 partitioning.

Materials

Protein of interest
0.1 M acetate buffer, pH 3.5 (see recipe)
0.5 M $NaNO_2$, made fresh
0.5 M NaCl

1. Dry two aliquots of the protein of interest in 1.5-ml microcentrifuge tubes and dissolve each in 0.1 ml of 0.1 M acetate buffer, pH 3.5.

   See Critical Parameters for guidelines on the total amount of protein to use.

   Nonidet P-40 (NP-40) detergent (0.1% v/v final) may be added to aid solubilization.

2. Add 0.1 ml of 0.5 M $NaNO_2$ to one tube. To the second tube (control), add 0.1 ml of 0.5 M NaCl. Incubate 3 hr at room temperature.

3. Detect the release of protein from GPI anchor by Triton X-114 partitioning and analyzing the reaction products (see Alternate Protocol 1).

   If the anchor is radiolabeled with fatty acids (UNIT 12.2), separation of lipid moiety may be used (see Support Protocol 3).

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SUPPORT PROTOCOL 3

SEPARATION OF LIPID MOIETY TO DETECT CLEAVAGE OF GPI-ANCHORED PROTEINS

If GPI-anchored proteins are radiolabeled with fatty acids, the efficacy of GPI anchor cleavage may be conveniently assessed by examining the released radiolabeled lipid products (see Fig. 12.5.1). In this protocol, radiolabeled proteins that have been cleaved by phospholipase or nitrous acid treatment are extracted with butanol. Released lipid moieties are extracted into the butanol phase, then analyzed and quantitated.

Materials

Radiolabeled protein, cleaved by phospholipase or nitrous acid treatment (see Alternate Protocol 2 and Basic Protocol 3)
Water-saturated *n*-butanol (see recipe)

1. Add 100 µl water to each of two 1.5-ml microcentrifuge tubes. To one tube, add 100 µl cleaved radiolabeled protein. To the second, add 100 µl control (uncleaved) protein.
2. Add 200 µl water-saturated *n*-butanol to each tube and vortex vigorously.
3. Microcentrifuge 30 sec at maximum speed, room temperature, to separate the two phases.
4. Remove the upper (butanol) phase to a fresh tube, and set aside. Add another 200 µl of water-saturated butanol to the original tubes.
5. Vortex and centrifuge as in steps 2 and 3 above.
6. Remove the second butanol phase and pool it with the first, retaining the lower (aqueous) phase for analysis.
7. Quantitate the percentage of lipid moieties released by the cleavage reactions by liquid scintillation counting of both butanol and aqueous phases. Compare counts recovered in butanol phases of the cleaved material to those recovered from control reactions to assess specific release.

To characterize released products fully, examine the butanol-extracted products by thin-layer chromatography (TLC) and compare to standards (Doering et al., 1990b). Products of phospholipase C digestion include diacylglycerol, alkyl-acyl glycerol, or ceramide, depending upon the original structure. Phosphatidylinositol (from nitrous acid treatment) or phosphatidic acid (released by phospholipase D) may also be analyzed and quantitated. This assay has been used to purify phospholipase activity (Hereld et al., 1986).

BASIC PROTOCOL 4

BASE HYDROLYSIS OF RADIOLABELED PROTEINS

For proteins radiolabeled with fatty acids, it is useful to ascertain whether incorporated fatty acids are actually part of a GPI structure or are present in amide linkage (*N*-myristoylation) or thioester linkage (as in palmitoylation of cysteine residues). This can be characterized by assessing the lability of the fatty acid linkage to various chemical treatments.

In this protocol, aliquots of radiolabeled proteins are resolved by electrophoresis and the various lanes are treated with KOH in methanol or hydroxylamine. Methanol and Tris·Cl buffer are used to treat the control lanes. The lanes are then processed for fluorography. If the chemical bonds in the protein are susceptible to these treatments, a radiolabeled signal will no longer be produced corresponding to the protein band of interest.

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Materials

Protein radiolabeled with fatty acid (*UNIT 12.2*)
0.2 M KOH in methanol
Methanol
1 M hydroxylamine·HCl, pH 7.5, made fresh (see recipe)
1 M Tris·Cl, pH 7.5 (*APPENDIX 2E*)

Additional reagents and equipment for one-dimensional SDS (*UNIT 10.1*) and staining of gels (*UNIT 10.5*)

1. Resolve five identical samples of protein radiolabeled with fatty acid by SDS-PAGE (*UNIT 10.1*). Include appropriate markers.
2. Cut apart the five gel lanes, and stain one to visualize protein and markers (*UNIT 10.5*).
3. Soak the other four lanes in one of the following solutions for 1 hr at room temperature:

   Lane 1: 0.2 M KOH in methanol
   Lane 2: methanol alone
   Lane 3: 1 M hydroxylamine·HCl, pH 7.5
   Lane 4: 1 M Tris·Cl, pH 7.5.

4. Soak the treated gel slices in water for three washes (10 min each).
5. Process gel pieces for autoradiography.

If bonds are susceptible to the treatments above, radiolabel from fatty acids will no longer produce a signal at the position of the protein band of interest. KOH in methanol cleaves thio- and oxyesters but not amide linkages. Hydroxylamine treatment cleaves thioesters only. Fatty acids that are part of a GPI moiety will be released by KOH in methanol, but not by hydroxylamine treatment. Methanol and Tris·Cl treatments serve as controls. Ceramide-containing anchors are not sensitive to mild base treatment (see Anticipated Results and Conzelmann et al., 1992).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Acetate buffer (pH 3.5), 0.1 M

Dissolve 0.82 g sodium acetate in 95 ml distilled water. Adjust pH to 3.5 with acetic acid, then bring volume to 100 ml with distilled water. Store indefinitely at room temperature.

GPI-PLC buffer

50 mM Tris·Cl, pH 8.0
5 mM EDTA
1% NP-40
Store indefinitely at 4°C

GPI-PLD buffer

50 mM Tris·Cl, pH 7.4
10 mM NaCl
2.5 mM $CaCl_2$
0.1% NP-40
Store indefinitely at 4°C

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Hydroxylamine·HCl (pH 7.5), 1 M

Dissolve 6.95 g hydroxylamine·HCl in 95 ml distilled water. Adjust pH to 7.5 with NaOH, then increase volume to 100 ml with water. Make fresh for each use.

PI-PLC buffer (for B. thuringiensis enzyme)

25 mM Tris acetate, pH 7.4
0.1% (w/v) sodium deoxycholate (Na-DOC)
Store indefinitely at 4°C

Tris-buffered saline (TBS)

10 mM Tris·Cl, pH 7.5
150 mM NaCl
Store indefinitely at room temperature

Water-saturated n-butanol

Mix equal volumes of distilled water and *n*-butanol. Cap tightly and shake vigorously. Let stand to allow phases to separate; the upper phase is the butanol. Store indefinitely at room temperature.

COMMENTARY

Background Information

Glycosyl phosphatidylinositol (GPI) membrane anchors are one mode whereby proteins are anchored to cell surfaces. These structures consist of a glycan bridge between phosphatidylinositol and phosphoethanolamine; the phosphoethanolamine is in amide linkage to the C-terminus of the protein (see Fig. 12.5.1). The glycan core of GPIs (shown schematically in Fig. 12.5.1) is remarkably conserved throughout evolution, although it is modified in many cell types by the addition of side chains. The lipid moieties of GPIs, however, are quite heterogeneous. This portion may consist of diacyl glycerol, alkyl acyl glycerol, *lyso*-acyl compounds, or ceramide. The hydrocarbon chains also vary in length and degree of saturation, and may be present as mixtures of species. GPI structures are reviewed in Englund (1993) and McConville and Ferguson (1993).

Much of our understanding of GPI anchors is derived from studies of the variant surface glycoprotein (VSG) of trypanosomes. Early work showed that VSG is associated with the membrane via a lipid moiety that contains dimyristoyl phosphatidylinositol (Cardoso de Almeida and Turner, 1983; Ferguson and Cross, 1984; Ferguson et al., 1985, 1986). Other investigations had shown that bacterial phospholipase C releases certain proteins from cell membranes (reviewed by Low, 1989). Together, these studies led to the definition of a class of proteins bearing a C-terminal glycolipid anchor.

Further investigations in this field have led to the elucidation of the pathway of GPI biosynthesis (reviewed by Doering et al., 1990a, and Field and Menon, 1991), identification of mutations in this pathway and several of the genes involved (Leidich et al., 1994, and reviews cited above), as well as the purification of a GPI-specific phospholipase C (GPI-PLC), and the development of anti-cross-reacting determinant (anti-CRD) antibodies as probes for GPIs. Phosphatidylinositol-specific phospholipase C (PI-PLC) and GPI-specific phospholipase D (GPI-PLD) are also useful in identification of GPI-anchored proteins.

A number of compounds found to inhibit GPI biosynthesis are reviewed in McConville and Ferguson (1993). One is mannosamine, which is taken up by cells and incorporated into the growing glycan, preventing completion of GPI precursor biosynthesis and subsequent anchorage (Pan et al., 1992; Ralton et al., 1993). This inhibitor prevents the cell-surface localization of proteins, as shown by Lisanti et al. (1991). PMSF and thiol alkylating reagents inhibit GPI anchor biosynthesis in vitro, as do several compounds affecting the synthesis of the mannose donor Dol-P-mannose (2-fluoro-2-deoxy-D-glucose and amphomycin; references in Lisanti et al., 1991). Data obtained from use of these compounds must be interpreted with care, as many also affect other forms of glycosylation, and their effects on GPIs are not always quantitative (see McConville and Ferguson, 1993). Although altered localization of a cell-surface protein of interest upon mannosamine treatment may indicate GPI anchorage, other methods must be used to confirm this, as such changes may be secondary to other

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adverse effects on the cell.

The protocols included here are designed to allow identification of GPI-anchored proteins without detailed structural analysis. Because these techniques are rather diverse, further explanatory information is outlined below.

Triton X-114 partitioning. Triton X-114 forms a clear micellar solution at low temperatures; the solution separates into two phases when warmed above 20°C, due to aggregation of detergent micelles (Bordier, 1981). When cellular material is extracted in Triton X-114 at low temperatures, the solution contains soluble proteins, integral membrane proteins, and GPI-anchored proteins. Centrifugation results in a pellet containing some of the GPI-anchored species as well as cellular components that are insoluble in nonionic detergents (Hooper and Bashir, 1991). When the detergent solution is warmed, amphiphilic proteins (including those with GPI anchors) associate with the detergent-enriched phase, while hydrophilic ones partition to the detergent-depleted phase. Some GPI-anchored proteins exhibit detergent insolubility that develops shortly after synthesis (Brown and Rose, 1992); this is thought to be due to aggregation of the anchored proteins with glycosphingolipids, possibly as a step in directed protein transport.

Once a GPI-containing protein is released from the lipid component of its anchor, it will no longer partition into the detergent-enriched phase. This alteration of partitioning behavior provides a rapid assay for the presence or absence of anchors. It may be used to monitor the changes in proteins produced by the other procedures.

Phospholipase digestion. Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) efficiently cleaves GPI anchors, releasing proteins in aqueous soluble forms. This enzyme also cleaves phosphatidylinositol (PI), *lyso*-PI, and PI-linked glycans. Proteins specifically released from membranes by PI-PLC have all been found to be GPI-anchored, making this an extremely reliable mode of analysis (for review see Ikezawa, 1991).

Several other specific phospholipases that effectively degrade only GPI species are also useful for analysis. These enzymes are generally more expensive than PI-PLC. One of these, initially purified from *Trypanosoma brucei*, is a GPI-specific phospholipase C (GPI-PLC). Another, present in mammalian serum (rat, rabbit, or human), is a GPI-specific phospholipase D (GPI-PLD). These enzymes are convenient tools for identifying the presence of a GPI anchor.

Release of a protein from its GPI anchor is followed by use of appropriate protein detection methods—e.g., enzyme activity assays, electrophoretic separation (*UNIT 10.1*) followed by protein staining (*UNIT 10.5*), and, if specific antibodies are available, immunoprecipitation or immunoblotting. Alternatively, treated cells may be studied for loss of a specific protein using immunofluorescence detection methods (Watkins, 1989), including fluorescence-activated cell sorting. Also, because release by PLC reveals a specific epitope (termed the cross-reacting determinant, or CRD), antibodies directed against this epitope may be employed to identify cleavage products resulting from PLC treatment (Support Protocol 2).

Lack of release may indicate absence of a GPI anchor, although some caution is required in interpreting negative results. Certain GPI molecules, bearing an additional fatty acid on inositol, are not susceptible to cleavage by either bacterial PI-PLC or trypanosomal GPI-PLC. Some proteins are partly sensitive, suggesting that only a fraction of these polypeptides bear anchors with acyl-inositol. Serum phospholipase D (GPI-PLD) cleaves GPI structures that contain acylated inositols, and is useful in their identification. However, it is not effective on membrane-bound proteins in the absence of detergent.

When intact cells are treated with phospholipase, partial or total resistance to cleavage can also occur due to inaccessibility of the protein to the enzyme, expression of a protein on the cell surface in both GPI-anchored and transmembrane-anchored forms, or tight association of a protein with a nonsusceptible protein on the cell surface (reviewed by Rosenberry, 1991).

Critical Parameters

Some details of these procedures will be affected by the method used to detect each protein under study. For example, specific quantities of starting materials are not provided because detection methods vary widely in terms of sensitivity. Also, for activity assays, the conditions of each procedure and whether the activity would survive such treatment must be considered. In general, plan to use two to ten times the amount of protein that would be reasonable for the mode of detection employed, depending upon the availability of material. This should allow for potential losses sustained during the procedure, as well as for the fact that proteins may be recovered in several fractions.

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Several GPI-anchored proteins, which may be useful control reagents for these protocols, are now available from Oxford GlycoSystems.

Triton X-114 partitioning provides a first hint of GPI anchorage, although transmembrane domains are also concentrated in the detergent phase of this partition. For example, if a protein is known from sequence data to have no membrane-spanning domain, its detection in the detergent phase of a Triton X-114 partition would be suggestive of lipid modification. In general, however, this method is most useful as a means of assessing release of a protein from its anchor, as shown by an alteration of partitioning behavior. Many of the other methods described specifically release proteins from their anchors to demonstrate GPI linkage (e.g., nitrous acid deamination or phospholipase digestion); Triton X-114 is a convenient tool for assessing such release. Triton X-114 often contains hydrophilic contaminants, and should be precondensed by several rounds of phase separation before use (Bordier, 1981).

Phospholipase digestion is useful for determining GPI linkage, and bacterial PI-PLC is perhaps the best enzyme to try initially for reasons of cost and efficacy. Although GPI-PLC is more specific, it is generally more expensive.

Some GPI anchors are not susceptible to cleavage by phospholipase C. These structures have an additional fatty acid covalently attached to the inositol of the anchor (Rosenberry, 1991). For this reason, failure of cleavage by PLC does not necessarily imply that a protein is not GPI-anchored. In such cases GPI-PLD may be useful, although this enzyme does not expose the epitope for anti-CRD detection. Extraction and analysis of radiolabeled lipid products from phospholipase digestions provides rapid assessment of cleavage. As mentioned earlier, radiolabeling the protein of interest is often helpful and permits less material and reagents to be used. In particular, if a protein may be radiolabeled with fatty acids, base hydrolysis is a helpful analytical tool, especially if lipid modification is suspected but other methods have yielded negative results.

Anticipated Results

Proteins anchored by GPI structures without acylated inositols should require detergent for solubilization. They may be extracted by Triton X-114 or remain in the cell pellet after such extraction (depending on the solubility properties of the protein). In a warmed Triton X-114 solution, anchored protein(s) partition into the detergent-enriched phase. These proteins can be chemically cleaved by nitrous acid deamination and enzymatically hydrolyzed by treatment with GPI-PLC, PI-PLC, or GPI-PLD. The protein cleaved with PLC will react with anti-CRD antibody, and appropriate lipid products will be generated from all cleavage reactions (Fig. 12.5.1). Radiolabel incorporated into the anchor as fatty acid is released by treatment with KOH in methanol, but not by hydroxylamine treatment, if the fatty acid is hydroxy-esterified to glycerol (as in diacyl or alkyl-acyl anchors). Some PI-PLC and nitrous acid-sensitive anchors (e.g., in yeast) have been found to contain ceramide instead of diacyl or alkyl-acyl glycerol. These anchors are resistant to mild base treatment but sensitive to conditions that hydrolyze amides (Conzelmann et al., 1992).

If the GPI anchor includes an acylated inositol, the above characteristics will hold true except that PLC treatment will not cleave the anchor. In these cases, nitrous acid deamination, GPI-PLD digestion, and radiolabel studies are used to characterize the linkage.

As mentioned above, removal of a GPI anchor may not alter the electrophoretic mobility of a protein at all, or may even decrease its apparent mobility. Size shifts may be used reliably to assess anchorage only after other methods (such as partitioning, anti-CRD reactivity, and specific radiolabel removal) have been used to ascertain that an observed change does correspond to anchor cleavage.

Time Considerations

In general, the methods described above are fairly rapid, with enzyme incubations or chemical reactions and subsequent partitioning requiring 1 to 2 hr. The time required for completion of these analyses depends primarily on the method used to detect the protein(s) of interest. For example, if a protein is radiolabeled in the fatty acid portion of the anchor, cleavage may be assessed by lipid extraction and scintillation counting in ≤1 hr. If immunoprecipitation or immunoblotting is used for product detection, analysis will be more lengthy.

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A useful laboratory manual of methods dealing with various post-translational modifications of proteins by lipids; many relate to GPI-linked proteins.

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This issue of Methods, titled "Covalent modification of proteins by lipids," contains two chapters specifically about GPIs—Doering et al., 1990b (see above) and Mayor and Menon (pp. 297-305)—as well as material on related topics.

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POST-TRANSLATIONAL MODIFICATION: PHOSPHORYLATION AND PHOSPHATASES

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CHAPTER 13
Post-Translational Modification: Phosphorylation and Phosphatases

INTRODUCTION

A polypeptide sequence deduced from a nucleic acid sequence tells only part of a protein's story: covalent modifications often play a key role in determining the final properties of a protein. Some modifications may be relatively constant over time; immediately obvious in this category is the important role played by disulfide bonds, which stabilize the folded structure of a protein and whose presence cannot be deduced from the sequence. Other modifications (e.g., N-linked glycosylation, covered in Chapter 12) may also affect protein properties in a relatively static sense by controlling (at least in part) solubility, antigenic structure, and, on occasion, the ability to interact with appropriate partner molecules. In other cases post-translational modifications are more dynamic: these are often of critical importance to protein function, directly controlling enzymatic activity or the ability to interact with partner molecules. Such modifications often take place on a time scale of minutes or even seconds. Foremost in this category is phosphorylation: changes in the phosphorylation state control the activity of a wide range of polypeptides, from transcription factors to soluble enzymes and cell-surface receptors (see *UNIT 13.1* for an overview of this topic).

The ability to detect the presence of phosphorylated amino acids and changes in phosphorylation status is essential in the study of phosphoprotein function. A method for labeling cells with inorganic phosphate, usually the first step in establishing whether a given polypeptide is phosphorylated in a particular cell type, is provided in *UNIT 13.2*. It is also important to distinguish among the different phosphoaminoacids. Methods for distinguishing between P-Ser, P-Thr, and P-Tyr are presented in *UNIT 13.3*. Phosphoproteins containing phosphotyrosine (but not other phosphoamino acids) can be detected by immunological means; this powerful and convenient technique is described in *UNIT 13.4*. Kinase reactions permit the addition of phosphate residues to polypeptides; it should also be kept in mind that the reverse reaction may be equally important as a means of controlling the activity of phosphoproteins. Similarly, phosphatases may be used analytically to demonstrate the presence of phosphorylated amino acid residues in phosphoproteins; this approach is presented in *UNIT 13.5*.

Production of antiphosphopeptide antibodies (i.e., antibodies that recognize phosphorylated peptides) is described in *UNIT 13.6*. This technology is an important tool for analysis of a protein's functional state, as these antibodies have unique specificity toward the cognate phosphorylated proteins and will not cross-react with unphosphorylated proteins or other phosphoproteins.

The assays for protein kinases are manifold and can include a variety of substrates. *UNIT 13.7* provides the strategies and protocols for assaying protein kinases using exogenous substrates. While the purpose of the unit is not to catalog all combinations of kinases and substrates, it provides specific examples of protein kinase assays that will assist in optimizing conditions for individual protein kinases.

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In many cases, the activities of kinases are contingent on the cellular context in which such activities are measured and on interactions with other proteins in the cell. In some cases prior activation of cell surface receptors is required to achieve full activation of the kinase(s) of interest and may determine the outcome of measuring the activity or the biochemical consequences of such kinase activity. Experiments with purified kinases may not accurately reflect these conditions, or the kinase(s) responsible may not be available in purified form. Under these circumstances, it is highly advantageous to conduct activity measurements in semi-intact or permeabilized cells or in subcellular fractions derived from them. *UNIT 13.8* describes different strategies for studying protein phosphorylation in permeabilized cells.

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Overview of Protein Phosphorylation

HISTORY

Phosphorylation is the most common and important mechanism of acute and reversible regulation of protein function. Studies of mammalian cells metabolically labeled with [^{32}P]orthophosphate suggest that as many as one-third of all cellular proteins are covalently modified by protein phosphorylation. Protein phosphorylation and dephosphorylation function together in signal transduction pathways to induce rapid changes in response to hormones, growth factors, and neurotransmitters. Most polypeptide growth factors (platelet-derived growth factor and epidermal growth factor are among the best studied; Heldin, 1995) and cytokines (e.g., interleukin 2, colony stimulating factor 1, and γ-interferon; Ihle et al., 1994) stimulate phosphorylation upon binding to their receptors. Induced phosphorylation in turn activates cytoplasmic protein kinases, such as raf, MEK, and MAP kinases (Marshall, 1995). Additionally, in all nucleated organisms, cell cycle progression is regulated at both the G1/S and the G2/M transitions by cyclin-dependent protein kinases (Doree and Galas, 1994).

Differentiation and development are also controlled by phosphorylation. Development of the R7 cell in the *Drosophila* retina (Simon, 1994) and of the vulva in *Caenorhabditis elegans* (Eisenmann and Kim, 1994) are both dependent on the function of receptor and cytoplasmic protein kinases. Finally, metabolism—in particular, the interconversion of glucose and glycogen and the transport of glucose—is regulated by phosphorylation (Cohen, 1985). Biologists of all stripes therefore find, often unexpectedly and occasionally reluctantly, that they must study protein phosphorylation in order to understand the regulation and function of their favorite gene and its product.

In the early 1940s, soon after their discovery that glycogen phosphorylase existed in two distinct forms in skeletal muscle, Carl and Gerty Cori identified a cellular activity that converted active phosphorylase *a* into a less active phosphorylase *b*. Initial studies of what they called the prosthetic-group removing (PR) enzyme suggested that the inactivation of phosphorylase *a* was mediated via proteolysis (Cori and Cori, 1945). More than ten years later, this converting enzyme was shown to be a protein phosphatase. Thus, a protein phosphatase was studied nearly 20 years prior to the identification of the first protein kinase. The finding that phosphorylase phosphatases were more widely expressed in tissues than phosphorylase *a* led to the current realization that these enzymes have a broad substrate specificity and regulate many physiological processes. The use of substrates other than phosphorylase *a* also identified several other protein serine/threonine phosphatases.

LABELING STUDIES

Protein phosphorylation is usually studied by biosynthetic labeling with ^{32}P-labeled inorganic phosphate ($^{32}P_i$; *UNIT 13.2*). This is intrinsically quite simple—the label is just added to growth medium. It is this step of an experiment, however, that makes many investigators the most nervous, given the perceived danger of radioactive exposure and the real danger of contamination of laboratory equipment with radioactivity. Neither problem is insurmountable. With proper shielding and technique, exposure of the investigator can be limited to the hands and contamination of the laboratory can be avoided. A general protocol for biosynthetic labeling with $^{32}P_i$ that maximizes incorporation and minimizes radioactive exposure of workers in the lab and contamination of lab equipment is described in *UNIT 13.2*. (For a general discussion of radiation safety consult Safe Use of Radioisotopes, *APPENDIX 2B*.)

Many protein kinases can be assayed by the transfer of radiolabel from [^{32}P]ATP to surrogate protein and peptide substrates. Synthetic peptide substrates have been particularly useful in establishing consensus motifs that define the substrate for protein kinases. By comparison, protein phosphatases show very low activity against synthetic phosphopeptides, greatly preferring the phosphoprotein substrates (Shenolikar and Ingebritsen, 1984). Therefore, attempts to define structural requirements for protein dephosphorylation using synthetic phosphopeptides have failed to establish the molecular basis for substrate specificity of the major cellular protein phosphatases.

The rate of ^{32}P incorporation into proteins in vivo, at either basal or hormone-stimulated levels, depends heavily on the rate of turnover of phosphate in the protein. For example, low phosphatase activity against specific sites or proteins will reduce the turnover of protein-bound phosphates and hinder their metabolic

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labeling in the intact cell. Conversely, physiological stimuli that increase phosphatase activity may facilitate metabolic labeling of proteins, allowing rapid replacement of endogenous "cold" phosphate with the radiolabel. Thus, ^{32}P incorporation by itself gives no indication about the extent of phosphorylation of a particular protein. As most phosphoproteins are phosphorylated on multiple sites, this also makes it difficult to correlate changes in protein function with ^{32}P labeling of a given protein in the intact cell.

SITES OF PHOSPHORYLATION

Most proteins are found to be phosphorylated at serine or threonine residues, and many proteins involved in signal transduction are also phosphorylated at tyrosine residues. These three hydroxyphosphoamino acids exhibit sufficient chemical stability at acidic pH that they can be recovered after acid hydrolysis and identified in a straightforward manner. Proteins that contain covalently bound phosphate at histidine, cysteine, and aspartic acid residues, either as phosphoenzyme intermediates or as stable modifications, have also been described. Each of these phosphoamino acids is chemically labile and impossible to study with the standard techniques used for the acid-stable phosphoamino acids. Indeed, they are often identified by inference or elimination. A technique for identifying phosphoserine, phosphothreonine, and phosphotyrosine by acid hydrolysis and two-dimensional thin-layer electrophoresis is described in *UNIT 13.3*. Techniques for analyzing acid-labile forms of protein phosphorylation are described in Ringer, 1991; Kamps, 1991; and Duclos et al., 1991.

Phosphotyrosine is not an abundant phosphoamino acid. Therefore, its detection in samples labeled with $^{32}P_i$ is often difficult, especially if the samples contain large quantities of proteins phosphorylated at serine residues or are contaminated with RNA. Detection of phosphotyrosine, as well as of phosphothreonine, can be enhanced considerably by incubation of gel-fractionated samples in alkali. This hydrolyzes RNA and dephosphorylates phosphoserine, allowing visualization of minor tyrosine- and threonine-phosphorylated proteins. A simple procedure for alkaline treatment is described in the alternate protocol of *UNIT 13.3*.

DETECTION OF UNLABELED PHOSPHOAMINO ACIDS

If a protein is modified by phosphorylation, identification of the phosphoamino acid can often be accomplished without resorting to biosynthetic labeling. For example, tyrosine phosphorylation can be studied because proteins containing this rare phosphoamino acid can be detected with great specificity and sensitivity by antibodies to phosphotyrosine (*UNIT 13.4*). By comparison, the quantitation of phosphoserine and phosphothreonine is more difficult and requires partial acid hydrolysis of the phosphoprotein and subsequent separation of phosphoamino acids by high-voltage electrophoresis on thin-layer cellulose acetate plates. Attempts to generate antibodies that recognize phosphoserine or phosphothreonine have failed to produce reagents with the required specificity and/or sufficient sensitivity to be useful. However, once the primary sequence around a phosphorylation site containing phosphoserine or phosphothreonine has been determined, it is possible to make antibodies against synthetic phosphopeptides modeled on these phosphorylation sites (Czernik et al., 1991). Such anti-phosphopeptide antibodies have been very useful tools for monitoring the phosphorylation of the parent protein at specific sites. The phospho-specific antibodies have also been used to inhibit the dephosphorylation of individual sites and demonstrate their role in protein function. Several recent studies have produced phospho-specific antibodies against phosphorylation sites predicted by the primary sequence of a protein. These reagents provide new insights into phosphorylations that control protein function.

More generally, because phosphorylation often alters the mobility of a protein during SDS–polyacrylamide gel electrophoresis and almost always alters its isoelectric point, the presence of phosphorylated residues in an unlabeled protein can be deduced from altered gel mobility after incubation of the protein with a phosphatase. Most phosphorylation sites are thought to reside near the surface of phosphoproteins. Therefore, they are equally accessible to phosphatases and proteases. Indeed, limited proteolysis often produces functional changes in phosphoproteins that closely mimic their in vitro dephosphorylation by phosphatase. As monitoring the release of radioactivity from a ^{32}P-labeled phosphoprotein does not distinguish between a phosphatase and a protease reaction, additional steps must to be taken to differentiate orthophosphate from small phosphopeptides. This is particularly important when dephosphorylation reactions are carried out in crude extracts (*UNIT 13.5*).

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PROTEIN KINASES

Protein kinases exhibit a strict specificity for phosphorylation of either serine/threonine or tyrosine residues. Yet these enzymes share structural homology in several domains such as the ATP-binding and catalytic sites, emphasizing their common function as phosphotransferases. A third group of protein kinases more closely resembles the serine/threonine kinases in their primary sequence but phosphorylate both serine/threonine and tyrosine residues. MEK, a MAP kinase activator (Crews and Erickson, 1992) and wee1, an inhibitor of cdc2 kinase (Parker et al., 1992) are examples of such dual-specificity protein kinases that phosphorylate threonine and tyrosine residues which are closely located in substrate proteins.

Kinases have been used in a number of ways to analyze protein phosphorylation. Mutations that abrogate ATP-binding inactive protein kinases. Such inactive kinases retain the ability to recognize substrates and therefore act as dominant-negative reagents to analyze the physiological function of kinases in the intact cell (Thorburn et al., 1994; Alberola-Ila et al., 1995). Cell-permeable compounds modeled on ATP also inhibit selected kinases in the intact cell.

Growth factors promote dimerization and activation of transmembrane receptor kinases. Mutant receptors that can dimerize in the absence of the natural ligand offer a novel approach to activate receptor kinases and initiate the cellular responses to growth factors (Spencer et al., 1993). Several cytoplasmic kinases are also activated by binding of intracellular second messengers. The allosteric activation of these kinases results from conformation changes that eliminate internal autoinhibitory interactions which silence the kinases in the absence of ligand. Deletion of the autoinhibitory sequences generates a constitutively active kinase that provides important insights into its physiological role (Planas-Silva and Means, 1992). Synthetic peptides modeled on the autoinhibitory sequences can also be used to selectively inhibit these kinases and reveal their role in controlling protein phosphorylation.

Still other kinases, which are not directly regulated by ligand binding, are activated by phosphorylation. Some of these kinases participate in cascades of sequential phosphorylations that amplify physiological signals. Substitution of the amino acids phosphorylated during the activation of these kinases with nonphosphorylated residues generates dominant-negative enzymes that can block the functions of such kinases in intact cells. Alternately, acidic amino acids may be substituted for phosphoamino acids to produce a constitutively active kinase (Brunet et al., 1994). Such reagents have been used to validate the role of protein kinases in specific phosphorylation events.

PROTEIN PHOSPHATASES

Many protein phosphatases also show a strict specificity for either phosphoserine/phosphothreonine or phosphotyrosine residues. However, unlike kinases, the serine/threonine and tyrosine phosphatases are not evolutionarily related and exhibit no primary sequence homology (Shenolikar and Nairn, 1991; Charbonneau and Tonks, 1992). Acid and alkali phosphatases can also dephosphorylate phosphoproteins in vitro but share no structural homology with protein phosphatases. A new family of phosphatases, such as cdc25 and CL100, are distantly related to the tyrosine phosphatases but dephosphorylate both phosphotyrosine and phosphothreonine in their target substrates. Mutation of an essential cysteine in tyrosine phosphatases has produced dominant-negative enzymes that have been shown to shield their substrates from dephosphorylation by endogenous phosphatases, thereby prolonging the biological effects of protein phosphorylation (Sun et al, 1993). Mutating several conserved amino acids can inactivate a serine/threonine phosphatase (Zhou et al, 1994), but the ability of such mutant phosphatases to modulate serine/threonine phosphorylation in cells has not yet been demonstrated.

A number of potent phosphatase inhibitors have been identified in recent years. Okadaic acid and several other toxins inhibit protein (serine/threonine) phosphatase 1 and 2A, and vanadate and phenylarsenoxide inhibit tyrosine phosphatase. These compounds have implicated reversible phosphorylation as a regulatory mechanism in many physiological processes (Cohen, 1989; Hardie et al., 1991; Shenolikar and Nairn, 1991; Shenolikar, 1994); under certain conditions, they may be the dominant regulators of these cellular processes. These phosphatase inhibitors can also be used to distinguish protein dephosphorylation from proteolysis in crude tissue extracts (Cohen, 1991). However, by far the most important contribution of these reagents has been that they have allowed assessment of the role played by phosphorylation in cellular processes where neither the identity of the phos-

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phoprotein involved nor that of the kinase(s) that regulates its function are known.

The units in this chapter describe techniques that detect protein phosphorylation and identify amino acids that have been covalently modified (*UNITS 13.2, 13.3, & 13.4*). The next step is to use phosphatases to dephosphorylate the substrate protein in vitro and address the functional relevance of the covalent modification (*UNIT 13.5*). Once the regulatory role of phosphorylation has been clearly established, the more sophisticated approaches discussed above can be used to to identify the specific kinases and phosphatases involved and thereby begin to elucidate the physiological mechanism that regulates the substrate in the intact cell.

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Labeling Cultured Cells with $^{32}P_i$ and Preparing Cell Lysates for Immunoprecipitation

This unit describes $^{32}P_i$ labeling and lysis of cultured cells to be used for subsequent immunoprecipitation of proteins. The procedure is suitable for insect, avian, and mammalian cells and can be used with both adherent and nonadherent cultures. The same general approach—biosynthetic labeling with $^{32}P_i$ in medium containing a reduced concentration of phosphate—can also be applied to bacteria and yeast; however, specific techniques to accomplish this are not presented here. This approach can also be used to label any cellular constituents with ^{32}P; in these cases the Basic Protocol must be modified beginning with step 8.

The first procedure described (see Basic Protocol) is $^{32}P_i$ labeling of adherent or nonadherent (e.g., hematopoietic) cells with subsequent lysis in a detergent buffer containing either Nonidet P-40 (NP-40), 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), or RIPA (RadioImmunoPrecipitation Assay buffer), a combination of NP-40, sodium deoxycholate, and SDS. More rigorous lysis conditions to be used for working with proteins that are difficult to solubilize are also described (see Alternate Protocol).

CAUTION: Unshielded ^{32}P will penetrate ~1 cm into flesh. Exposure to the skin and eyes is, therefore, of concern. Gloves and protective eyeware should always be worn when handling significant amounts of ^{32}P. A 1-in.-thick (2.5-cm) Plexiglas shield, tall enough to look through when seated or standing comfortably, should be used when handling samples containing ^{32}P.

LABELING CULTURED CELLS WITH $^{32}P_i$ AND LYSIS USING MILD DETERGENT

BASIC PROTOCOL

The first six steps of this protocol describe culturing and labeling procedures for cultures of adherent cells; modifications appropriate for nonadherent cells are described in alternate steps. The remaining steps describe lysis of cells in detergent buffer to prepare the sample for immunoprecipitation. For a more detailed discussion of mammalian cell culture conditions and reagents, see *APPENDIX 3C*.

Materials

Cell culture to be labeled
Labeling medium: phosphate-free tissue culture medium (e.g., DMEM; *APPENDIX 3C*) supplemented with the usual concentration of serum or serum dialyzed against phosphate-free saline, 37°C
500 mCi/ml $H_3{}^{32}PO_4$ in HCl (carrier-free; ICN)
Tris-buffered saline (TBS, see recipe) or PBS (see recipe), cold
Mild lysis buffer or RIPA lysis buffer (see recipes)

1-in.-thick Plexiglas shield (*APPENDIX 2B*)
Plugged, aerosol-resistant pipet tips
Plexiglas box (*APPENDIX 2B*), warmed to 37°C
Screw-cap microcentrifuge tubes
Plugged disposable pipet or disposable one-piece transfer pipet
Rubber policeman

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Sorvall refrigerated centrifuge with SM-24 rotor and rubber adaptors, refrigerated microcentrifuge, or equivalent, 4°C

Plexiglas sheet (10 × 10 × ¼–in.) or Plexiglas tube holder, 4°C (*APPENDIX 2B*)

Additional reagents and equipment for cell culture (*APPENDIX 3C*) and gel electrophoresis (*UNIT 10.1*)

NOTE: All culture incubations are performed in a humidified 37°C, 10% CO_2 incubator unless otherwise specified.

Label the cell culture

1. Culture the cells to be labeled to an appropriate stage of growth.

   Phosphate transport is maximal in rapidly growing cells. Therefore, except in those cases where phosphorylation of a protein in quiescent cells is to be examined, cells to be labeled should be subconfluent (adherent cells) or at less than maximal density (nonadherent cells). It is useful to change the medium to fresh growth medium 3 to 18 hr prior to labeling.

   Brief cultivation of cells in low-phosphate medium (to reduce the phosphate pool by starvation prior to labeling) is of minimal value.

For adherent cells

2a. Remove growth medium by aspiration. Wash away any residual phosphate-containing medium by adding 37°C labeling medium supplemented with serum, but lacking the label, and removing the wash medium by aspiration.

   Phosphate-free DMEM is routinely used for labeling medium because RPMI has a very high concentration of phosphates.

3a. Add prewarmed labeling medium to cultures, using 0.5 to 1 ml per 35-mm dish, 1 to 2 ml per 50-mm dish, or 2 to 4 ml per 100-mm dish of adherent cells.

For nonadherent cells

2b. Gently centrifuge the culture 1 min at 1800 × *g* and aspirate the medium away from the cell pellet. Resuspend the cells in labeling medium supplemented with serum but lacking the label. Centrifuge again for 1 min at 1800 × *g* and remove the medium.

3b. Add 2 ml prewarmed labeling medium per 10^7 cells and transfer to an appropriate size petri dish.

4. Working behind a Plexiglas shield, use a micropipettor with plugged, aerosol-resistant pipet tips to add $^{32}P_i$ to a final concentration of 0.1 to 2 mCi/ml.

   Use of plugged, aerosol-resistant pipet tips will minimize contamination of the micropipettor. $^{32}P_i$ is usually shipped in HCl and is generally sterile, so it is not necessary to sterilize the labeling medium after adding the radioisotope. Additionally, except in the rare cases when steady-state labeling with ^{32}P is desired, the labeling interval is usually so short (<6 hr) that microbial growth resulting from added isotope is undetectable. Therefore, addition of ^{32}P to cells can be performed on the lab bench rather than in a tissue culture hood.

5. Place dishes in a warmed Plexiglas box, and put the box in the incubator.

   Labeling for 1 to 2 hr is usually sufficient, but cells will tolerate as much as 2 mCi/ml for 6 hr and lower concentrations (0.1 to 0.5 mCi/ml) for 18 hr.

Wash and lyse the cells

6. At the end of the labeling period, carry the labeled cells, still in the Plexiglas box, into the cold room. Place the cells behind a Plexiglas shield.

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For adherent cells

7a. Take the dish out of the box and remove the labeling medium manually, using either a plugged disposable pipet or a disposable one-piece plastic Pasteur transfer pipet. Discard the medium and pipet as radioactive waste.

Labeling medium should be removed without using a vacuum aspirator. Vacuum aspiration generates radioactive aerosols and leaves a radioactive film on the equipment.

8a. Wash cells once with 2 to 10 ml cold TBS. Remove the wash buffer manually, as in step 7, and discard as radioactive waste.

The uptake of phosphate is efficient and often a majority of the added $^{32}P_i$ is found within the labeled cells. This necessitates continued shielding of the labeled cells following removal of the labeling medium.

9a. Add lysis buffer to cells, using 0.3 ml per 35-mm dish, 0.6 ml per 50-mm dish, or 1.0 ml per 100-mm dish of adherent cells. Dislodge adherent cells by scraping with a rubber policeman, but leave the lysate in the dish. Incubate 20 min at 4°C. With a rubber policeman, scrape the lysate of adherent cells to the side of the dish and transfer lysate to a screw-cap microcentrifuge tube using a disposable one-piece plastic transfer pipet.

If a low background of nonspecific contaminants is critical, use RIPA lysis buffer. If maintenance of enzymatic activity or the structure of protein complexes is critical, use a milder lysis buffer containing either CHAPS or NP-40 as the only detergent. If complete solubilization of the cells and denaturation of the protein is desired, use SDS for lysis (see Alternate Protocol).

For nonadherent cells

7b. Take the dish out of the box, transfer the cells to a screw-cap centrifuge tube, pellet them by centrifugation (1 min at 1800 × *g*), and remove the medium.

Do not use a vacuum aspirator to remove medium.

8b. Resuspend pelleted cells gently in a small volume of cold TBS, transfer to a screw-cap microcentrifuge tube, and pellet the cells by microcentrifuging 1 min at 1800 × *g*.

Continue to use shielding with cells and lysate.

9b. Add 0.5 to 1 ml lysis buffer per 10^7 cells and resuspend the pellet by gentle agitation with a disposable plastic Pasteur pipet. Incubate 20 min at 4°C.

Choose the lysis buffer as in annotation to step 9a.

10. Cap the tube, and clarify the lysate by centrifuging 30 min at 26,000 × *g* (17,000 rpm in Sorvall SM-24 rotor), 4°C.

Use an ice bucket with a sheet of Plexiglas over it, a chilled Plexiglas tube holder, or a prechilled centrifuge rotor to transport the tube of lysate.

It is best to half-fill the tube to prevent spilling. A Sorvall SM-24 rotor with rubber adaptors for microcentrifuge tubes is ideal for clarifying lysates. Alternatively, a refrigerated microcentrifuge can be used for 30 min at maximum speed. Do not use a nonrefrigerated microcentrifuge in the cold room because the centrifuge will warm to 20°C during a 30-min spin.

Lysates prepared with RIPA buffer often become viscous due to lysis of nuclei. If this occurs, increase the time of centrifugation to 90 min, or add 50 µl of fixed Staphylococcus aureus bacteria (Pansorbin, Calbiochem) in RIPA buffer to the lysate prior to centrifugation. Either modification will cause the solubilized DNA to pellet.

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11. After centrifugation, transfer the supernatant (lysate) to a new tube and discard the tube and pellet in radioactive waste.

If the pellet is very viscous, so that it is impossible to remove the supernatant cleanly, suck part of the pellet into a micropipet tip, lift the viscous material out of the tube, and discard it in the radioactive waste. The residual liquid in the tube is the supernatant and can be used for immunoprecipitation.

12. Analyze the labeled lysate using gel electrophoresis (*UNIT 10.1*), immunoprecipitation, or protein purification. Carry out all analytical procedures at 4°C using adequate shielding.

ALTERNATE PROTOCOL

LYSIS OF CELLS BY BOILING IN SDS

Some proteins, such as eukaryotic RNA polymerase II, are difficult to solubilize with mild lysis buffer or RIPA lysis buffer, and some analytical procedures use antibodies that recognize epitopes exposed only in denatured proteins. In these cases, it is useful to solubilize labeled cells completely in SDS and then adjust the composition of the lysate solution to match that of RIPA buffer for immunoprecipitation. To avoid the formation of spurious disulfide bonds, lysis and washing during immunoprecipitation are carried out in the presence of fresh 1 mM dithiothreitol (DTT). The protocol describes the procedure for adherent cells; modifications for working with nonadherent cells are described as alternate steps.

Additional Materials *(also see Basic Protocol)*

SDS lysis buffer (see recipe)
RIPA correction buffer (see recipe)
Immunoprecipitate wash buffer (see recipe)
Fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem; optional)
Boiling water bath

1. Label and wash cells (see Basic Protocol steps 1 to 7).

2a. *For adherent cells:* Add SDS lysis buffer using 0.1 ml for a 35-mm dish, 0.25 ml for a 50-mm dish, or 0.5 ml for an 100-mm dish. Immediately scrape off the dish with a rubber policeman and transfer the cell lysate to a screw-cap microcentrifuge tube.

2b. *For nonadherent cells:* Vortex briefly to loosen the cell pellet, add 1 ml SDS lysis buffer per 5×10^7 cells and vortex again.

3. Boil the samples 2 to 5 min, then add 4 vol RIPA correction buffer and mix well.

4. Clarify the cell lysate by centrifuging 90 min at 26,000 × *g* (17,000 rpm in Sorvall SM-24), 4°C, or at maximum speed in a refrigerated (4°C) microcentrifuge.

Lysate may also be clarified by adding 50 µl fixed Staphylococcus aureus and centrifuging 30 min at 26,000 × g, 4°C.

5. Carry out immunoprecipitation as usual using immunoprecipitate wash buffer for washes.

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REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Immunoprecipitate wash buffer for boiled sample

RIPA lysis buffer (see recipe)
1 mM DTT, added fresh

DTT is added from a 1 M stock solution stored at –20°C.

Mild lysis buffer

10 mM 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) or 1% (w/w) Nonidet P-40 (NP-40)
0.15 M NaCl
0.01 M sodium phosphate, pH 7.2
2 mM EDTA
50 mM sodium fluoride
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

CHAPS is a milder detergent than NP-40, but yields precipitates with a higher background and may solubilize some proteins less efficiently.

Sodium vanadate stock solution can be stored in plastic at room temperature.

Phosphate-buffered saline (PBS)

Dissolve the following in 800 to 900 ml H_2O:
8 g sodium chloride (136.8 mM final)
0.2 g potassium chloride (2.5 mM final)
0.115 g dibasic sodium phosphate (anhydrous; 0.8 mM final)
0.2 g monobasic potassium phosphate (anhydrous; 1.47 mM final)
0.1 g calcium chloride (anhydrous; 0.9 mM final)
0.1 g magnesium chloride hexahydrate (0.5 mM final)
Add H_2O to 1 liter
Dispense 50- or 100-ml aliquots into glass bottles
Autoclave and store indefinitely at room temperature

RIPA (RadioImmunoProtection Assay) correction buffer for boiled sample

1.25% (w/w) Nonidet P-40 (NP-40)
1.25% (w/v) sodium deoxycholate
0.0125 M sodium phosphate, pH 7.2
2 mM EDTA
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
50 mM sodium fluoride
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

Sodium vanadate stock solution can be stored in plastic at room temperature.

RIPA (RadioImmunoPrecipitation Assay) lysis buffer

1% (w/w) Nonidet P-40 (NP-40)
1% (w/v) sodium deoxycholate
0.1% (w/v) SDS
0.15 M NaCl
0.01 M sodium phosphate, pH 7.2
2 mM EDTA

continued

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50 mM sodium fluoride
0.2 mM sodium vanadate added fresh from 0.2 M stock solution
100 U/ml aprotinin (Trasylol, Pentex/Miles)
Store buffer without vanadate at 4°C up to 1 year

Sodium vanadate stock solution can be stored in plastic at room temperature.

SDS lysis buffer

0.5% (w/v) SDS
0.05 M Tris·Cl, pH 8.0 (*APPENDIX 2E*)
1 mM DTT, added fresh

SDS and Tris·Cl solutions can be made in advance and stored at room temperature. DTT is added from a 1 M stock solution stored at –20°C.

Tris-buffered saline (TBS)

8 g sodium chloride (136.8 mM final)
0.38 g potassium chloride (5.0 mM final)
0.1 g calcium chloride (anhydrous; 0.9 mM final)
0.1 g magnesium chloride hexahydrate (0.5 mM final)
0.1 g dibasic sodium phosphate (anhydrous; 0.7 mM final)
800 to 900 ml H_2O
Add 25 ml 1 M Tris·Cl, pH 7.4 (*APPENDIX 2E*)
Add H_2O to 1 liter
Dispense 50- or 100-ml aliquots into glass bottles
Autoclave and store indefinitely at room temperature

COMMENTARY

Background Information

The extent to which a protein becomes radiolabeled via biosynthetic labeling with $^{32}P_i$ depends on the rate of transport of phosphate into the cells being labeled, abundance of the protein, stoichiometry of phosphorylation of the protein, and rate of phosphate turnover in the protein. The rate of turnover of the protein itself is less important because phosphate in protein usually turns over at a much faster rate than does the protein. Most of the phosphate taken up by cells is not incorporated into phosphoproteins—the vast majority is incorporated into phospholipid, RNA, and DNA. The efficiency of incorporation of $^{32}P_i$ into proteins is therefore low—only ~1% of incorporated radioactivity is found in phosphoproteins.

An obvious and immediate question is how many cells and how much isotope should be used. The answer is that it depends on the protein being studied. If the protein is abundant (i.e., it constitutes 0.5% to 1% of total cellular protein) and highly phosphorylated (i.e., it contains 1 mole of phosphate per mole of protein), it can be labeled by incubating 10^6 cells with 0.2 to 0.5 mCi $^{32}P_i$ in 2 ml labeling medium for 1 hr. In contrast, if the protein is rare or non-abundant, it may be necessary to label 10^7 cells with 5 to 10 mCi of $^{32}P_i$ in 3 to 5 ml labeling medium for 4 to 6 hr. A pilot experiment can be useful: if 50 cpm are recovered in the protein of interest, use more cells and more isotope; if 200,000 cpm are recovered in the protein of interest, use fewer cells with less isotope.

Critical Parameters

Incorporation of $^{32}P_i$ during biosynthetic labeling is greatest if labeling is done in medium lacking any phosphate except the added radioisotope. Labeling can also be accomplished at a somewhat reduced efficiency in complete growth medium. Phosphate-free medium is commercially available but can also be easily formulated in-house by omitting sodium and potassium phosphate from a recipe and replacing them with either sodium chloride or potassium chloride or both. Serum dialyzed against phosphate-free saline can be used instead of complete serum to further reduce the level of phosphate in labeling medium. Dialysis of serum against phosphate-free saline reduces but never entirely eliminates the phosphate in serum. Washing or rinsing cells in reduced-phosphate medium just prior to labeling significantly increases labeling efficiency, but starvation of cells by incubation in reduced-phosphate medium prior to labeling is of very limited value.

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Labeling cells with ^{32}P almost certainly induces radiation damage in the labeled cells and will affect or arrest cell-cycle progression. If phosphorylation of the protein under study varies during the cell cycle, labeling with ^{32}P may alter its phosphorylation.

At the end of the labeling interval, the radioactive medium should be discarded, except in experiments where labeled virions or labeled secreted proteins are being studied. Labeling medium should be removed *without* using a vacuum aspirator. Vacuum aspiration generates radioactive aerosols and leaves a radioactive film inside the aspirator hose. As a result the hose can become an intense source of radiation exposure. It is best to use a plugged disposable pipet or disposable plastic Pasteur pipet for removing labeling medium. The medium and pipet should be discarded immediately as radioactive wastes.

In general, labeled cells are lysed and subjected to gel electrophoresis, immunoprecipitation, or protein purification. If the labeled cells are to be centrifuged, they should be transferred to capped, disposable centrifuge tubes. Tubes should be no more than half full if they are to be spun in a fixed-angle rotor; tubes that are too full may leak radioactive lysate and contaminate the centrifuge. Screw-cap microcentrifuge tubes are ideal for centrifuging lysates. If large volumes are being handled, multiple partially filled tubes are preferable to tubes filled to the top.

An ice bucket can be used for storage and transport of samples to keep the samples cold and provide considerable shielding. Radiation exposure from the tops of the tubes can be minimized by covering the ice bucket with a ¼-in. Plexiglas sheet.

RadioImmunoPrecipitation Assay (RIPA) buffer (Brugge and Erikson, 1977) is the lysis buffer of choice because it solubilizes proteins well, gives a low background of nonspecific proteins, and is tolerated by most antigens and antibodies. It does however denature some antigens and disrupt some protein:protein complexes. If 1% (w/w) Nonidet P-40 (NP-40) is used as the only detergent, most of these problems are solved without increasing background unacceptably (Sefton et al., 1980). Some workers like to use digitonin as an extremely mild detergent for cell lysis. Digitonin sometimes is tricky and idiosyncratic, giving erratic results and high backgrounds during immunoprecipitation. 3[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS; Chen et al., 1990) and Brij 96 (Osman et al., 1992) are mild detergents that appear to give more reproducible results.

Besides the usual concern about proteolysis following cell lysis, the cells must be handled carefully to prevent protein phosphorylation and dephosphorylation following lysis of the cells. Both problems can be minimized by proper formulation of the lysis buffer and by keeping the sample at 4°C. Inclusion of 2 mM EDTA in the lysis buffer will minimize phosphorylation in the lysate by chelating both Mg^{2+} and Mn^{2+}, which are essential for protein kinase activity. Addition of phosphatase inhibitors to the lysis buffer will reduce dephosphorylation significantly. For example, 50 mM sodium fluoride is used to inhibit serine and threonine phosphatases, and 0.2 mM sodium vanadate is used to inhibit all known tyrosine phosphatases. Sodium vanadate appears to lose its effectiveness if it is stored diluted in lysis buffer, so it should be added fresh each time. The concentrated stock solution should be stored in plastic at room temperature.

Anticipated Results

This protocol can be used to label phosphoproteins or other phosphorylated cellular constituents. Phosphoproteins will contain ~1% of incorporated label.

Time Considerations

Proteins can and should be labeled biosynthetically with $^{32}P_i$ and isolated by immunoprecipitation in a single day. As is the case with all ^{32}P-labeled samples, it is important to work quickly because the specific activity of the sample declines 5% per day. If it is absolutely necessary to stop during the immunoprecipitation, it is best to leave the samples as precipitated pellets after aspiration of the wash buffer. Such samples can be stored at 4°C or –20°C overnight. Freezing the cell lysate tends to increase the background. Frozen lysates should be reclarified by centrifugation prior to immunoprecipitation.

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Phosphoamino Acid Analysis

It is often valuable to identify the phosphorylated residue in a protein. In the case of proteins phosphorylated at serine, threonine, or tyrosine, this is readily accomplished by partial acid hydrolysis in HCl followed by two-dimensional thin-layer electrophoresis of the labeled phosphoamino acid (Basic Protocol). Phosphothreonine and phosphotyrosine are more stable to hydrolysis in alkali than are RNA and phosphoserine. Therefore, mild alkaline hydrolysis of protein samples can be used to enhance the detection of phosphothreonine and phosphotyrosine (Alternate Protocol).

Although this procedure can be carried out with a protein eluted from a preparative gel and concentrated by trichloroacetic acid (TCA) or acetone precipitation, it is most easily accomplished by transfer of the protein of interest to a PVDF membrane. This technique is obviously not ideal if the protein being studied does not transfer efficiently.

NOTE: Wear gloves and use blunt-end forceps to handle membranes.

ACID HYDROLYSIS AND TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF PHOSPHOAMINO ACIDS

BASIC PROTOCOL

The protein to be acid hydrolyzed is transferred to a PVDF membrane using the same technique used for immunoblotting or for microsequencing. It is useful, but not absolutely essential, to keep the filter wet following transfer. Following acid hydrolysis, phosphoamino acids are separated by two-dimensional thin-layer electrophoresis. Because electrophoresis equipment differs considerably in design, the details of the assembly and placement of the plate are not discussed here. It is assumed that a suitable apparatus is available for use by an experienced operator. Electrophoresis conditions are described for using the HTLE 7000 (CBS Scientific). They are almost certainly not correct for other equipment and will need to be altered according to the equipment manufacturer's directions.

Materials

^{32}P-labeled phosphoprotein (*UNIT 13.2*)
India ink solution: 1 µl/ml India ink in TBS (see recipe)/0.02% (v/v) Tween 20, pH 6.5 (prepare fresh or store indefinitely at room temperature); *or* radioactive or phosphorescent alignment markers
6 M HCl (*APPENDIX 2E*)
Phosphoamino acid standards mixture (see recipe)
pH 1.9 electrophoresis buffer (see recipe)
pH 3.5 electrophoresis buffer (see recipe)
0.25% (w/v) ninhydrin in acetone in a freon (aerosol, gas-driven) atomizer/sprayer
PVDF membrane (Immobilon-P, Millipore)
110°C oven
Screw-cap microcentrifuge tubes
20 cm × 20 cm × 100 µm glass-backed cellulose thin-layer chromatography plate (EM Sciences)
Large blotter: two 25 × 25–cm layers of Whatman 3MM paper sewn together at the edges, with four 2-cm holes that align with the origins on the TLC plate
Glass tray or plastic box
Whatman 3MM paper
Thin-layer electrophoresis apparatus (e.g., HTLE 7000, CBS Scientific)
Fan

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Small blotters: 4 × 25–cm, 5 × 25–cm, and 10 × 25–cm pieces of Whatman 3MM paper
50° to 80°C drying oven
Sheets of transparency film for overhead projector

Additional reagents and equipment for SDS-PAGE (*UNIT 10.1*), electroblotting onto PVDF membranes (*UNIT 10.7*), and staining membranes (*UNIT 10.8*)

Prepare sample

1. Run radiolabeled phosphoprotein on a preparative SDS-polyacrylamide gel (*UNIT 10.1*).

2. Transfer proteins electrophoretically to a PVDF membrane (*UNIT 10.7*). Wash the membrane several times with water. Do not let the membrane dry.

   These washes remove buffer and detergent.

3. Locate the band of interest by staining the membrane 5 to 10 min in 30 to 50 ml India ink solution with shaking until bands are detectable, *or* by wrapping the membrane in plastic wrap, applying radioactive or phosphorescent alignment markers, and performing autoradiography.

4. Excise the piece of membrane containing the band of interest with a clean razor blade. Rewet the piece of membrane with methanol for 1 min and then rewet it in >0.5 ml water. Place the piece of membrane paper in a screw-cap microcentrifuge tube.

   Keep the excised piece as small as possible.

Hydrolyze sample

5. Add enough 6 M HCl to submerge the piece of membrane. Screw the cap on the tube tightly and incubate 60 min in 110°C oven.

6. Let cool. Microcentrifuge 2 min at maximum speed. Transfer the liquid hydrolysate to a fresh microcentrifuge tube and dry with a Speedvac evaporator.

   Drying takes ~2 hr. Simultaneous drying of the hydrolysate and deblocked oligonucleotides in NH_4OH must be avoided, as this will generate a cloud of ammonium chloride that will collect in the centrifuge tube and render the hydrolysate unsuitable for thin-layer electrophoresis.

7. Dissolve the sample in 6 to 10 µl water by vortexing vigorously. Microcentrifuge 5 min at maximum speed.

Prepare plate for first-dimension electrophoresis

8. Spot 25% to 50% of the sample, in 0.25- to 0.50-µl aliquots, on one origin of a 20 cm × 20 cm × 100 µm glass-backed cellulose thin-layer chromatography plate (see Fig. 13.3.1 for arrangement of samples). Between each application, dry the sample spot with compressed air delivered through a Pasteur pipet plugged with cotton.

   Use long, thin plastic micropipet loading tips for loading, and do not let the tip touch the plate.

   Four samples can be analyzed simultaneously. The complete hydrolysate can be spotted on a single origin, but some streaking in the first dimension may be observed due to overloading. This problem can be avoided by using a fraction of the sample.

9. Spot 1 µl nonradioactive phosphoamino acid standards mixture (containing phosphoserine, phosphothreonine, and phosphotyrosine) on top of each sample in 0.25- to 0.50-µl aliquots as above.

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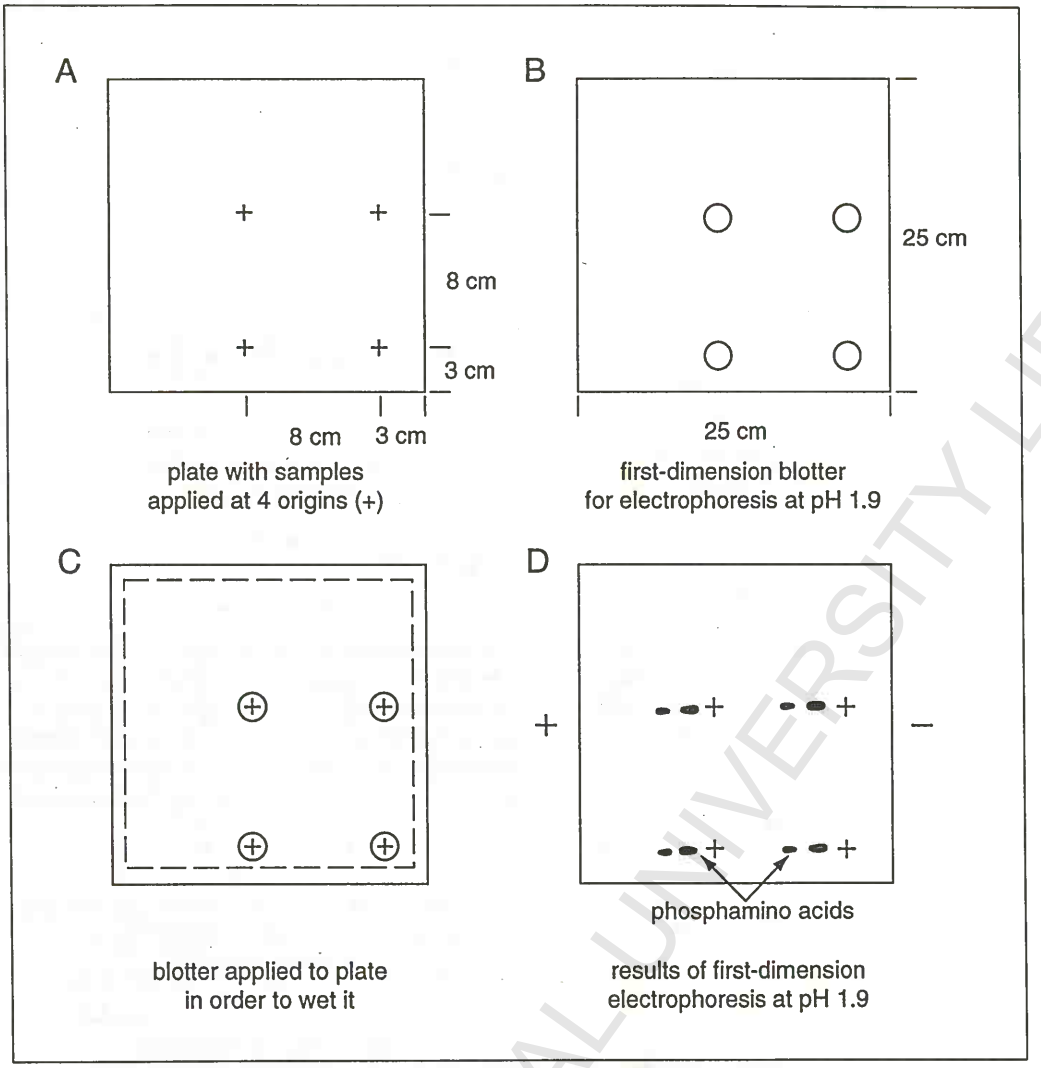

Figure 13.3.1 First-dimension electrophoretic separation of phosphoamino acids at pH 1.9. (**A**) Positions of the four origins on a single 20 × 20–cm plate; (**B**) blotter used for wetting the plate with pH 1.9 electrophoresis buffer; (**C**) placement of the blotter on the plate (underneath; indicated by dashed outline); and (**D**) orientation of the plate between the + and – electrodes with the positions of the phosphoamino acids after electrophoresis.

10. Wet the large blotter (with four holes) by submerging it in pH 1.9 electrophoresis buffer in a large glass tray or plastic box. Briefly allow the excess buffer to drain off. Lower the wet blotter onto the prespotted plate with the origins on the plate in the centers of the four holes in the blotter (Fig. 13.3.1). Press on the blotter gently to achieve even wetting of the cellulose and concentration of the samples. When the plate is uniformly wet, remove the blotter.

    The blotter should be quite damp but not sopping wet. Excess buffer can be wicked off onto filter paper.

    Areas of the plate that are too dry can be seen through the blotter and will appear to be whiter than the rest of the plate. If this happens, dab the blotter with a Kimwipe wetted with pH 1.9 electrophoresis buffer. If there are puddles of buffer on the plate, let them dry before carrying out electrophoresis.

11. Place the thin-layer plate in the electrophoresis apparatus and overlap 0.5 cm of the right and left sides of the plate with wicks made of Whatman 3MM paper. If the

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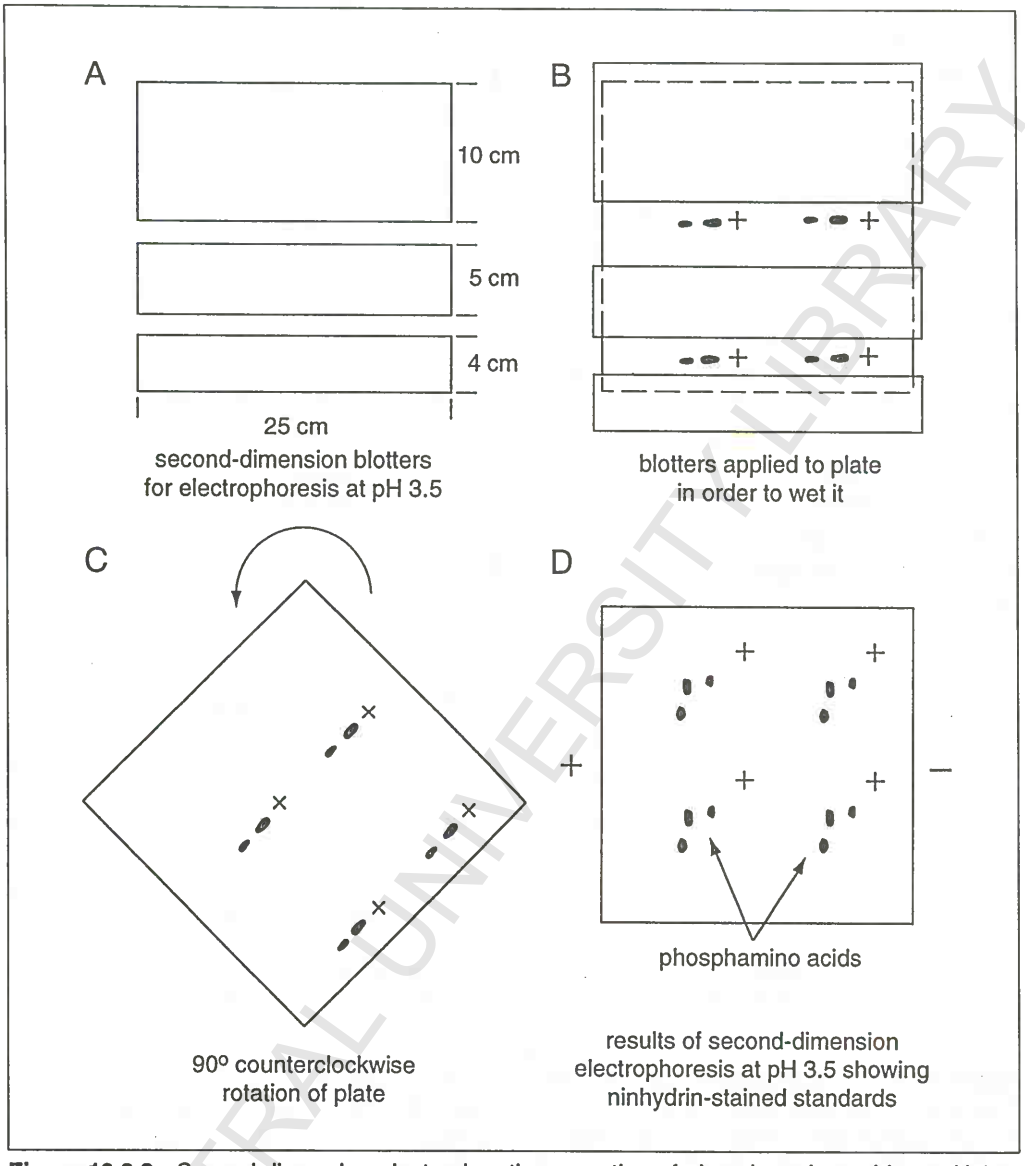

Figure 13.3.2 Second-dimension electrophoretic separation of phosphoamino acids at pH 3.5. **(A)** The three pieces of Whatman 3MM paper used for wetting the plate with pH 3.5 electrophoresis buffer; **(B)** proper placement of the blotters on the plate (underneath; indicated by dashed outline); **(C)** reorientation of the plate for electrophoresis in the second dimension; and **(D)** orientation of the plate between the + and – electrodes with the position of the phosphoamino acids after electrophoresis.

apparatus has an air bag, be sure to inflate it. Close the cover and start electrophoresis. With an HTLE 7000, double-thickness Whatman 3MM wicks, and a plate with four samples, electrophorese 20 min at 1.5 kV.

For the HTLE 7000 apparatus, use folded-over Whatman 3MM wicks that are 20 cm wide (the same as the plate) and not overly wet. Overly wet wicks will flood the plate and cause sample diffusion.

For other electrophoresis, apparatuses the appropriate duration of electrophoresis can be determined empirically by examining the rate of migration of the phosphoamino acid standards.

12. Following electrophoresis, remove the plate and quickly air dry with a fan without heating.

It takes ~20 min to dry the plate.

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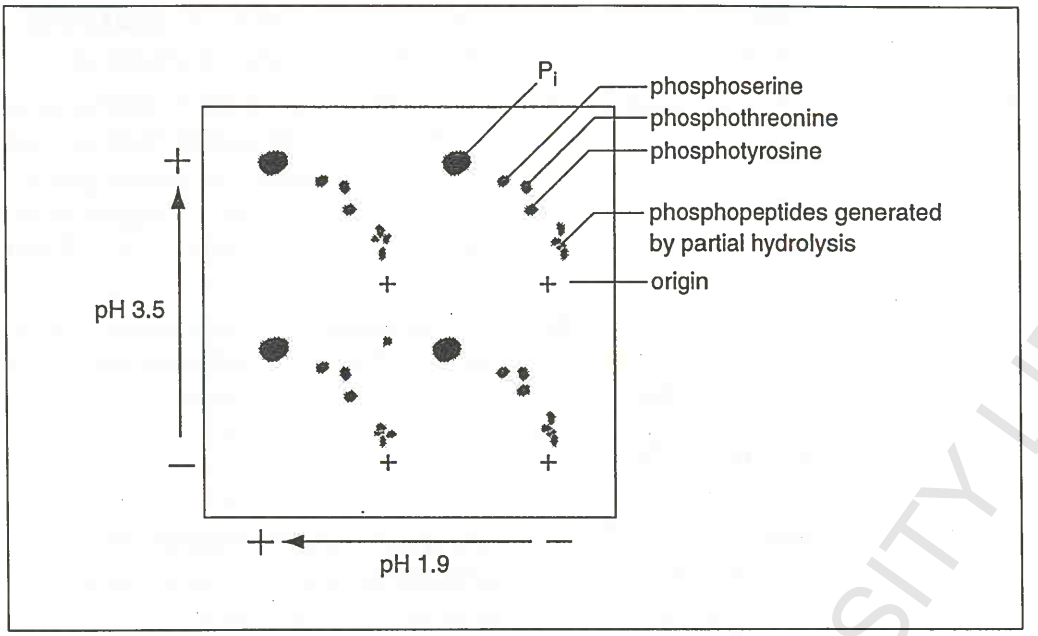

Figure 13.3.3 Hypothetical autoradiogram of a two-dimensional separation. Four samples of acid-hydrolyzed, ^{32}P-labeled proteins are applied at the origins, one in each of the four quandrants. This diagram shows the origins, the directions of electrophoresis, the positions of phosphoserine, phosphothreonine, and phosphotyrosine, the position of P_i, and the position of partially hydrolyzed fragments of the proteins for the upper right-hand sample. Every protein generates different partial hydrolysis peptide fragments.

Perform second-dimension electrophoresis

13. Wet the small blotters in pH 3.5 electrophoresis buffer and use them to wet the plate using the method described in step 10 to achieve even wetting without puddling (Fig. 13.3.2).

    After electrophoresis at pH 1.9, phosphoamino acids are present as a streak extending from the origin towards the + electrode. Blotters are not applied directly over the phosphoamino acids to prevent sample blurring or smearing.

14. Remove the blotters, rotate the plate 90° counterclockwise, and electrophorese 16 min at 1.3 kV in pH 3.5 electrophoresis buffer if using the HTLE 7000 apparatus.

15. At the end of the electrophoresis run, remove the plate and dry 20 to 30 min in an oven at 50° to 80°C. When dry, spray with 0.25% ninhydrin in acetone, then reheat in the oven 5 to 10 min to visualize the phosphoamino acid standards.

16. Place radioactive or phosphorescent alignment marks on the plate and autoradiograph with an intensifying screen overnight to 10 days at –70°C.

17. Following autoradiography, trace the alignment markers and the stained phosphoamino acid markers onto a transparent sheet used for overhead projectors. Save this template. Align the film with the plate and identify radioactive phosphoamino acids (Fig. 13.3.3).

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ALTERNATE PROTOCOL

ALKALI TREATMENT TO ENHANCE DETECTION OF TYR- AND THR-PHOSPHORYLATED PROTEINS BLOTTED ONTO FILTERS

Because phosphothreonine and phosphotyrosine are much more stable to hydrolysis in alkali than RNA or phosphoserine, detection of proteins containing phosphothreonine and phosphotyrosine can often be enhanced by mild alkaline hydrolysis of gel-fractionated samples. Although this technique was first developed for the treatment of fixed polyacrylamide gels, it is much more easily performed with proteins that have been first transferred to a PVDF membrane.

Alkaline hydrolysis does not preclude subsequent phosphoamino acid analysis. A band from a blot that has been treated with alkali can be excised and subjected to acid hydrolysis as described in the Basic Protocol.

Additional Materials (also see Basic Protocol)

1 M KOH
TN buffer: 10 mM Tris·Cl (pH 7.4 at room temperature)/0.15 M NaCl
1 M Tris·Cl, pH 7.0 at room temperature (*APPENDIX 2E*)

Covered plastic container (e.g., Tupperware box)
55°C oven or water bath

1. Run radiolabeled phosphoprotein on a preparative SDS–polyacrylamide gel and transfer proteins electrophoretically to a PVDF membrane (see Basic Protocol, steps 1 and 2).

   A nylon membrane may be used in place of a PVDF membrane, but in that case, the bands cannot subsequently be analyzed by acid hydrolysis, as nylon membrane will dissolve in 6 M HCl.

2. Wash membrane thoroughly with water: three 2-min incubations in 1 liter water are sufficient.

   These washes remove buffer and detergent.

3. Incubate membrane 120 min at 55°C in an oven or water bath in sufficient 1 M KOH to cover the filter in a covered Tupperware container.

4. Discard KOH. Wash membrane and neutralize remaining KOH by rinsing once for 5 min in 500 ml TN buffer, once for 5 min in 500 ml of 1 M Tris·Cl (pH 7.0), and twice for 5 min in 500 ml water. Wrap the membrane in plastic wrap and autoradiograph overnight with flashed film and an intensifying screen at –70°C.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

pH 1.9 electrophoresis buffer

50 ml 88% formic acid (0.58 M final concentration)
156 ml glacial acetic acid (1.36 M final concentration)
1794 ml H_2O
Store indefinitely in a sealed bottle at room temperature

pH 3.5 electrophoresis buffer

100 ml glacial acetic acid (0.87 M final concentration)
10 ml pyridine [0.5% (v/v) final concentration]
10 ml 100 mM EDTA (0.5 mM final concentration)
1880 ml H_2O
Store indefinitely in a sealed bottle at room temperature

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Phosphoamino acid standards mixture

Prepare a solution of phosphoserine, phosphothreonine, and phosphotyrosine (Sigma) in water at a final concentration of 0.3 μg/ml each. Store in 1-ml aliquots indefinitely at –20°C.

Tris-buffered saline (TBS)

For 100 ml, add 10 ml solution A to 89 ml H_2O. While stirring rapidly, add 1 ml solution B slowly, drop by drop. Filter sterilize and store at 4°C.

Solution A:
80 g/liter NaCl
3.8 g/liter KCl
2 g/liter Na_2HPO_4
30 g/liter Tris base
Adjust pH to 7.5
Filter sterilize
Store at –20°C

Solution B:
15 g/liter $CaCl_2$
10 g/liter $MgCl_2$
Filter sterilize
Store at –20°C

Make certain that the stock TBS solution is mixed well just before use by bringing the solution into and out of a sterile 10-ml pipet several times.

COMMENTARY

Background Information

Phosphoamino acid analysis by the two-dimensional electrophoretic technique described in the Basic Protocol was first carried out with proteins isolated by elution from unfixed SDS-polyacrylamide gels (Hunter and Sefton, 1980). However, this technique is laborious, especially if it involves grinding up pieces of high-percentage acrylamide gels, and the yields can be disappointing. Additionally, because the eluted protein must be precipitated in the presence of a carrier protein, spotting the whole sample on a single origin usually yields a badly smeared pattern. The grind-and-elute technique is, however, advantageous with proteins that are very refractory to electrophoretic transfer to PVDF membranes.

The alkaline treatment of protein described in the Alternate Protocol was first developed by Jon Cooper and Tony Hunter, who treated fixed gels with KOH (Cooper and Hunter, 1981). The original technique is tricky because the gel becomes extremely sticky during incubation with KOH and swells. Additionally, the manipulations needed to recover proteins from the gel following treatment are very involved because the proteins are contaminated with products of the hydrolysis of polyacrylamide.

Critical Parameters and Troubleshooting

It is essential to use PVDF membranes to immobilize proteins for acid hydrolysis rather than nylon or nitrocellulose membranes, both of which dissolve in 6 M HCl. Proteins immobilized on either PVDF or nylon membranes may be subjected to alkaline hydrolysis with KOH (Contor et al., 1987), but nitrocellulose membranes are not suitable. Proteins immobilized on nylon cannot subsequently be analyzed by acid hydrolysis because nylon is dissolved by 6 M HCl.

Two-dimensional thin-layer electrophoresis is required for unambiguous identification of phosphorylated residues, as some spots after one-dimensional electrophoresis do not represent pure species. For example, uridine monophosphate, which is generated during acid hydrolysis of RNA (a frequent contaminant of phosphoproteins), comigrates with phosphotyrosine during one-dimensional electrophoresis at pH 3.5.

Streaking of the sample in the first dimension is a symptom of overloading, either with the phosphoprotein itself or with contaminants in the sample. This problem can be corrected by loading less sample. Streaking in the second dimension is usually the result of problems with wetting or running the plate and cannot be corrected by loading less sample.

Some batches of blotting paper contain calcium, which interferes with electrophoresis of phosphoamino acids (probably by precipitating them). Inclusion of EDTA in the pH 3.5 buffer alleviates this problem. In the author's experience Whatman 3MM paper is quite reliable; other blotting papers are probably suitable as well.

This unit calls for glass-backed cellulose thin-layer plates rather than the plastic-backed

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variety, which are lighter and less expensive. This is because plastic-backed plates can under some circumstances cause sample streaking. They are, however, probably satisfactory for most experiments. If use of plastic-backed plates results in streaking, try glass-backed plates to see if that corrects the problem.

Anticipated Results

To detect a phosphoamino acid by autoradiography, a minimum of 10 cpm must be spotted and the plate exposed for a week with flashed film and an intensifying screen. Only 15% to 20% of the radioactivity in a phosphoprotein is recovered as phosphoamino acids. The majority is present as $^{32}P_i$, which is released by dephosphorylation of phosphoamino acids, with the remainder being peptide products resulting from partial acid hydrolysis. As a result, the thin-layer plates will contain a number of radioactive spots that are not phosphoamino acids (see Fig. 13.3.3). Partial hydrolysis products remain near the origin during electrophoresis at pH 1.9, but exhibit some mobility at pH 3.5 (see Fig. 13.3.3). After two-dimensional electrophoresis, they are found above the origin and below the phosphoamino acids. $^{32}P_i$ has a high mobility at both pH 1.9 and pH 3.5 and is found in the upper left-hand corner of each quadrant of the plate (see Fig. 13.3.3). Because of these additional radioactive spots, it is essential to localize internal phosphoamino acid standards by staining with ninhydrin.

Time Considerations

After the preparative gel has been run and the protein transferred to the membrane, isolation of the membrane fragment containing the protein, followed by acid hydrolysis, takes <2 hr. Two to three hours are required for drying the sample with a Speedvac evaporator. First- and second-dimension electrophoresis and staining the internal phosphoamino acid standards takes no more than ~2 hr. Overnight autoradiographic exposure with flashed film and an intensifying screen is usually sufficient; however, exposures can be carried out for up to 10 days before the background from the screen becomes objectionable.

Detection of phosphorylated protein after alkali treatment, as described in the Alternate Protocol, is a very quick procedure requiring little more time than it takes to carry out the alkaline hydrolysis. In general, samples of this sort can be detected after overnight exposure with flashed film and an intensifying screen at –70°C.

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Key Reference

Kamps, M.P. and Sefton, B.M. 1989. Acid and base hydrolysis of phosphoproteins bound to Immobilon facilitates the analysis of phosphoamino acids in gel-fractionated proteins. *Anal. Biochem.* 176:22-27.

Discusses all of the variables involved in subjecting filter-bound proteins to acid and base hydrolysis.

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Detection of Phosphorylation by Immunological Techniques

Phosphorylation of unlabeled proteins can be detected using immunological or enzymatic techniques (*UNIT 13.5*). Anti-phosphotyrosine antibodies are used with immunoblots to detect tyrosine phosphorylation. These antibodies can be detected by ^{125}I-labeled protein A (Basic Protocol) or enhanced chemiluminescence (ECL; Alternate Protocol).

IMMUNOBLOTTING WITH ANTI-PHOSPHOTYROSINE ANTIBODIES AND DETECTION USING [^{125}I]PROTEIN A

BASIC PROTOCOL

Initial determination of whether a protein is phosphorylated on tyrosine is carried out by immunoblotting with antibodies to phosphotyrosine. This technique is often superior to the use of biosynthetic labeling with $^{32}P_i$ because of its sensitivity and low background, and it can be used to detect tyrosine phosphorylation of proteins in tissues that do not incorporate $^{32}P_i$ efficiently.

Materials

Protein sample: Cultured cells, tissue, lysate, or immunoprecipitate
Transfer buffer containing 100 µM sodium vanadate
Blocking buffer (see recipe)
Anti-phosphotyrosine antibody: 2 µg/ml rabbit polyclonal anti-phosphotyrosine (UBI) *or* mouse monoclonal anti-phosphotyrosine—e.g., py20 (Leinco, ICN Biomedicals, Zymed, Transduction Laboratories) or 4G10 (UBI)—in blocking buffer
TN buffer: 10 mM Tris·Cl (pH 7.4 at room temperature)/0.15 M NaCl (*APPENDIX 2E*)
TNA solution: TN buffer (recipe above)/0.01% (v/v) sodium azide (store at room temperature)
NP-40 wash solution: 0.05% (v/v) Nonidet P-40 (NP-40)/TNA solution (store at room temperature)
0.5 µCi/ml ^{125}I-labeled protein A (30 mCi/mg, ICN) in blocking buffer
India ink solution: 1 µl India ink in TBS (*UNIT 13.3*)/0.02% (w/v) Tween 20, pH 6.5 (prepare fresh or store indefinitely at room temperature; optional)

Plastic container with lid (e.g., Tupperware box)
Blotting paper

Additional reagents and solutions for SDS-PAGE (*UNIT 10.1*) and fluorography (*UNIT 10.2*)

NOTE: Wear gloves and use blunt-end forceps to handle membrane.

1. Dissolve cultured cells, tissue, or lysate immunoprecipitate in an equal volume of 2× SDS sample buffer and boil 5 min.

   Lysates or immunoprecipitates in sample buffer can be stored at –70°C indefinitely. Storage >1 week at –20°C will lead to deterioration. Cells or tissues can be lysed by other means, but care must be taken to prevent phosphorylation or dephosphorylation from occurring following cell lysis. This can be accomplished by using 50 mM sodium fluoride to inhibit serine and threonine phosphatases, 0.2 mM sodium vanadate to inhibit tyrosine phosphatases, and 2 mM EDTA to inhibit kinases. Viscosity resulting from DNA can be reduced somewhat by boiling the sample 5 min and/or by shearing the sample by passing it five to ten times through a 22-G needle and five to ten times through a 27-G needle.

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2. Electrophorese sample on an SDS-polyacrylamide gel (*UNIT 10.1*). Transfer proteins to a membrane suitable for immunoblotting using transfer buffer that contains 100 μM sodium vanadate.

   The lysate from ~10^5 cells can be analyzed in a single 0.5-cm wide lane of a 1-mm-thick SDS-polyacrylamide slab gel.

   Sodium vanadate should be included in the transfer buffer to prevent tyrosine dephosphorylation.

3. Incubate membrane in 30 to 50 ml blocking buffer ≥30 min at room temperature.

   Use of Blotto or other blocking mixtures containing dry milk is unsuitable because anti-phosphotyrosine antibodies bind to a number of the protein constituents of milk. Blocking buffer can be reused several times.

4. Incubate the membrane in anti-phosphotyrosine antibody 60 to 120 min at room temperature with occasional agitation.

   This step is most conveniently done in a covered plastic box using 30 to 50 ml antibody solution for a typical 15 × 15–cm blot. Although the volume of antibody solution seems large, it reduces the background. The antibody solution can be saved in the plastic box, stored at 4°C for at least a month, and reused up to five to ten times.

5. Remove the membrane from the staining box and wick off excess liquid with blotting paper. In a new plastic box, wash the membrane twice for 10 min with 50 ml TBSA, twice for 10 min with 50 ml NP-40 wash solution, and twice for 5 min with 50 ml TBSA. Discard washes.

6. Incubate membrane in 30 to 50 ml of 0.5 μCi/ml ^{125}I-labeled protein A in blocking buffer 60 min at room temperature with gentle agitation in a covered plastic box.

   ^{125}I-labeled protein A is used to detect bound antibody.

7. Remove the membrane and wick off excess liquid with blotting paper. Wash the membrane as described in step 5 in a clean plastic box.

   The blotting paper and first wash should be discarded as radioactive wastes. The amount of radioactivity in the subsequent washes should not be great, and it is generally acceptable to pour them down the drain.

   Protein A solution can be stored at least 1 month at 4°C and reused ~3 to 5 times.

8. If desired, stain the membrane 5 to 10 min with 30 to 50 ml India ink solution until bands are detectable. Wash the membrane with water to remove excess ink.

9. Remove excess moisture with blotting paper, wrap in plastic wrap, attach radioactive or fluorescent alignment markers, and expose by fluorography using an intensifying screen and preflashed film for 18 hr to 10 days.

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DETECTION OF BOUND ANTIBODIES BY ENHANCED CHEMILUMINESCENCE (ECL)

ALTERNATE PROTOCOL

Bound anti-phosphotyrosine antibodies can also be detected using enhanced chemiluminescence (ECL). This technique uses a second antibody coupled to horseradish peroxidase to detect anti-phosphotyrosine antibodies. The bound second antibody is visualized by ECL and the image is captured on film. The technique is faster than that employing [^{125}I]-labeled protein A (Basic Protocol) because it requires autoradiographic exposure of only minutes rather than days. It also avoids the use of radioactivity. However, results obtained with the two techniques are not always identical and exhibit quantitative differences. Additionally, if immunoprecipitated proteins are being analyzed, the secondary antibody may stain the precipitating antibodies intensely, making detection of proteins of a size similar to those of the heavy and light chains of immunoglobulin difficult.

The technique is fundamentally the same as that using [^{125}I]-labeled protein A, except that azide must be omitted from all solutions because it interferes with chemiluminescence chemistry. This makes storage and reuse of antibodies more difficult. Additionally, the technique appears to work somewhat better with nitrocellulose membranes than with Immobilon-P. Therefore, handling of the membranes requires somewhat more delicacy.

Additional Materials (also see Basic Protocol)

Blocking buffer (see recipe) *without* azide
0.05% (v/v) Nonidet P-40 (NP-40)/TN buffer (see Basic Protocol)
Horseradish peroxidase–conjugated secondary antibody: anti-rabbit or anti-mouse antibody diluted 1/1000 to 1/2000 in blocking buffer *without* azide
ECL detection reagents A and B (Amersham)
Luminol reagent for ECL detection (Amersham)
Oxidizing reagent for ECL detection (Amersham)

Nitrocellulose membrane
Plastic container slightly larger than the membrane
Plastic sheet protector

NOTE: The reagents for ECL may be purchased as a kit, the Enhanced Chemiluminescence Western Blotting Detection System, from Amersham.

NOTE: Wear gloves and use blunt-end forceps to handle membrane.

Prepare membrane

1. Prepare sample in 2× SDS-sample buffer (see Basic Protocol, step 1) and electrophorese on an SDS-polyacrylamide gel. Transfer proteins to a nitrocellulose membrane using a transfer buffer that contains 100 µM sodium vanadate.
2. In a plastic container, incubate membrane in blocking buffer without azide ≥30 min at room temperature.

Expose membrane to antibodies

3. Incubate membrane in 30 to 50 ml anti-phosphotyrosine antibody 60 to 120 min at room temperature with occasional agitation.

   The anti-phosphotyrosine antibody can be either polyclonal rabbit serum or mouse monoclonal antibodies.

4. In a clean plastic container, wash the membrane twice for 10 min with TN buffer, twice for 10 min with 0.05% NP-40/TN buffer, and twice for 5 min with TN buffer. Discard the washes.

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5. In a plastic container, incubate the membrane in 30 to 50 ml of horseradish peroxidase–conjugated secondary antibody 60 min at room temperature with gentle agitation.

   Use a secondary antibody appropriate for the primary antibody.

6. Remove membrane and wash it in a clean plastic container as described in step 4.

Detect bound antibodies with ECL

7. In a plastic container only slightly larger than the membrane, mix equal volumes of the Luminol reagent and the oxidizing reagent for ECL detection.

   Amersham recommends that 0.125 ml of each reagent be used per square centimeter of membrane. This is probably excessive with large blots.

8. Put the membrane, with the side containing protein facing up, into the mixed reagents. Agitate gently 60 sec.

9. Remove the blot, wick off excess moisture, and place it, protein-side up, in a sheet protector.

   Alternatively, place the blot on a sheet of transparency film used for overhead projectors and cover with a second sheet of transparency film.

10. In the darkroom, expose the blot to X-ray film.

    Exposure times can range from 15 sec to 30 min.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Blocking buffer

5% (w/v) BSA
1% (w/v) hen ovalbumin
10 mM Tris·Cl, pH 7.4 at room temperature
0.15 M NaCl
0.01% (w/v) sodium azide
Store ≤6 months at 4°C

For enhanced chemiluminescence, omit the sodium azide.

COMMENTARY

Background Information

Immunoblotting with antibodies to phosphotyrosine is an extremely useful technique for the study of tyrosine phosphorylation. It appears to be very specific. Although there is a report that antibodies to phosphotyrosine recognize phosphohistidine (Frackelton et al., 1983), nonspecific cross-reactivity is rarely seen. It is possible to be misled, however, if a large amount of a single protein is subjected to immunoblotting. For example, nonspecific staining of molecular weight standards is often observed. This almost certainly does not indicate that they contain phosphotyrosine. Rather, it illustrates the fact that the primary antibodies and the secondary detection reagents exhibit a low level of nonspecific binding that can be detectable if a large amount of a single protein is present in a sample.

Critical Parameters

Not all anti-phosphotyrosine antibodies give the same staining patterns for immunoblots of the same sample. Each antibody appears to react with some proteins more strongly than with others. This presumably reflects the amino acid sequence surrounding the phosphotyrosine residue, but the cause has not been examined carefully. Correspondingly, a given anti-phosphotyrosine antibody will not react equally strongly with all sites of tyrosine phosphorylation. For example, rabbit polyclonal anti-phosphotyrosine antibodies raised against phosphoTyr-Gly-Ala react more strongly with

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phosphorylated Tyr-394 in the Lck tyrosine protein kinase than they do with phosphorylated Tyr-505. The differential reactivity of phosphotyrosine should be kept in mind when drawing quantitative conclusions.

Diluted polyclonal anti-phosphotyrosine antibodies can be reused several times. Their properties, however, change subtly with reuse. The staining of some bands becomes less intense and the staining of others becomes apparently more prominent. This may be a consequence of preferential removal of high-affinity antibodies during early uses. It should not be a problem with monoclonal antibodies.

Analysis of immunoprecipitated proteins by immunoblotting can be problematic. The reagent used to detect the staining antibody on the membrane will often bind to the heavy and light chains of the precipitating antibodies. This occurs to a varying degree with [^{125}I]-labeled protein A, which under some circumstances can bind detectably to the heavy chain of rabbit antibodies even after they have been denatured on a membrane. It is a greater problem when second antibodies reactive with the species of antibody used for immunoprecipitation are used as detection reagents. For example, horseradish peroxidase–conjugated anti-mouse antibodies will, in general, bind strongly to both the heavy and light chains of mouse monoclonal antibodies used for immunoprecipitation. This can complicate the use of enhanced chemiluminescence (ECL) as a means for analyzing immunoblots of immunoprecipitated proteins.

Immunoprecipitates can be run on nonreducing gels to cause interfering antibody bands to migrate near the top of the gel. Unreduced IgG runs as a 150-kDa band. Nonreducing sample buffer is the same as SDS sample buffer except that 50 mM iodoacetamide replaces the reducing agents 2-mercaptoethanol (2-ME) and DTT. The mobility of many, but not all, proteins is altered profoundly under nonreducing conditions and the suitability of nonreducing gel electrophoresis must be evaluated carefully for each case. It is often difficult to analyze reduced and nonreduced samples on the same gel because 2-ME, which is present in most sample buffers at very high concentrations, diffuses rapidly and can reduce nonreduced samples, even in the presence of 50 mM iodoacetamide.

The ECL technique (Alternate Protocol) is very rapid and sensitive. It is unclear, however, whether it exhibits the same linearity of response as does autoradiography with [^{125}I]-labeled protein A. Immunoblotting should be used with caution for quantitative comparisons. For accurate quantitation, it is essential to determine the linearity of the detection system by measuring the signal obtained from multiple known dilutions of a standard sample.

Not all proteins exhibit altered gel mobility when they undergo phosphorylation or dephosphorylation. Lack of effect of phosphatase digestion on the mobility of a protein is therefore not evidence of lack of phosphorylation.

Anticipated Results

Immunoblotting with anti-phosphotyrosine antibodies is very sensitive. Even the extremely low levels of tyrosine phosphorylated proteins present in unactivated lymphoid cells can be detected when total lysates of cells are analyzed by gel electrophoresis and immunoblotting.

Time Considerations

Immunoblotting with enhanced chemiluminescence is the most rapid technique described in this unit, because staining can be accomplished in less than a day and bound antibodies visualized within 30 min. Staining with [^{125}I]-labeled protein A can also be accomplished in a single day, but autoradiography usually requires 18 to 100 hr.

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Detection of Phosphorylation by Enzymatic Techniques

Reversible protein phosphorylation has been increasingly recognized as an important mechanism for regulating physiological processes in both plant and animal cells. There are a number of techniques to demonstrate the presence of covalently bound phosphate in proteins. Metabolic labeling of cells with ^{32}P and subsequent isolation of the ^{32}P-labeled protein is the most direct method for demonstrating protein phosphorylation. This approach is difficult when the specific polypeptides involved in the physiological phosphorylation event have not been well characterized or when these proteins are present in such low abundance in the cell that significant incorporation of [^{32}P]phosphate cannot be achieved. Metabolic labeling of proteins (*UNIT 13.2*) may also be compromised by a slow turnover of the protein-bound phosphate in the intact cell. As the extent of phosphorylation of given protein reflects the relative activities of the protein kinases and phosphatases that act on it, knowledge of physiological stimuli that modulate the protein function might be expected to establish the experimental conditions which increase the metabolic labeling of the substrate protein. In the absence of this information it may be difficult to optimize protein phosphorylation in the intact cell. This situation can be further exacerbated by the lack of experimental tools, such as monospecific antibodies or affinity probes, that permit the rapid isolation of the substrate protein and preserve its phosphorylation.

If significant ^{32}P-labeling of the desired protein cannot be easily demonstrated, a different approach must be taken. Recent studies have utilized one or more phosphatases to reverse existing covalent modifications in proteins, resulting in changes in the function or activity of the substrate. In vitro dephosphorylation by one or more commercially available phosphatases has implicated phosphorylation as a regulatory mechanism in many physiological processes. These studies have been undertaken using cell extracts and subcellular fractions as well as partially purified proteins. The methodologies discussed in this unit can be used to detect protein phosphorylation where actual incorporation of [^{32}P]phosphate into a specific polypeptide cannot be demonstrated.

The overall strategy is to first examine the functional effects elicited by nonspecific acid or alkaline phosphatases that dephosphorylate many phosphoproteins in vitro (Basic Protocol 1 and Alternate Protocol 1). Nonspecific phosphatases isolated from plant and animal tissues show significant activity against a wide variety of phosphoester-containing compounds, including many phosphoproteins. These enzymes reverse all phosphorylations whether they occur as phosphotyrosine, phosphothreonine, phosphoserine, or phosphohistidine so they can define the regulatory consequence of protein dephosphorylation without necessarily identifying the specific phosphoamino acid. Protein phosphatases that selectively hydrolyze phosphoserine and phosphothreonine or phosphotyrosine residues can then be used to identify the functionally important covalent modification. Additional protocols describe digestion of phosphoproteins with a protein serine/threonine phosphatase (Basic Protocol 2) and protein tyrosine phosphatase (Alternate Protocol 2). A support protocol has been included to identify the radiolabel as $^{32}P_i$ based on its ability to form a complex with ammonium molybdate.

Protein dephosphorylation can be analyzed as the loss of [^{32}P]phosphate from metabolically labeled proteins following their separation by SDS-PAGE. Some phosphoproteins also show changes in their electrophoretic migration following the loss of covalently bound phosphate. This can provide an additional monitor of protein dephosphorylation that can be used even in the absence of metabolic labeling. Finally, measurements of protein function (e.g., opening or closing of ion channels, binding of nuclear factors to specific DNA elements, or altered enzyme activity) can define the consequences of

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dephosphorylation even when little information is available about the specific phosphopeptides involved.

BASIC PROTOCOL 1

DIGESTION OF PHOSPHOPROTEINS WITH NONSPECIFIC ACID PHOSPHATASES

Potato acid phosphatase shows a broad substrate specificity and hydrolyzes many metabolites containing phosphoester bonds. This enzyme shows comparatively low activity against phosphoproteins. Moreover, metabolites present in cell extracts or some subcellular fractions may inhibit its ability to dephosphorylate protein substrates. In this protocol, a sample containing a phosphoprotein is digested with potato acid phosphatase and analyzed for evidence of structural and functional changes.

Materials

Sample containing 100 to 200 µg total protein
50 mM piperazine-*N*,*N*′-bis(2-hydroxypropanesulfonic acid) (PIPES), pH 6.0
Sephadex G-25 column (optional)
PIPES/2-ME or PIPES/DTT buffer, pH 6.0: 50 mM PIPES containing 15 mM 2-mercaptoethanol or 1 mM dithiothreitol (prepare fresh)
Potato acid phosphatase
2× SDS-PAGE sample buffer: 50 mM Tris·Cl/0.4 M glycine (pH 8.3)/0.2% (w/v) SDS
100 mM sodium pyrophosphate or other general phosphatase inhibitor
90°C water bath or heating block

Additional reagents and equipment for electrophoresis (*UNIT 10.1*)

1. Prepare a protein sample containing 100 to 200 µg total protein. Remove inhibitory metabolites or particulate material.

   If the substrate is particulate, soluble contaminants can be removed by washing 2 to 3 times with 50 mM PIPES, pH 6.0. Inhibitory metabolites can be removed by dialyzing against the same buffer, or if the substrate is a soluble protein, it can be desalted on a Sephadex G-25 column.

2. Incubate the sample in 100 µl PIPES/2-ME or PIPES/DTT, pH 6.0, for 10 min at 30°C.

   The pH optimum for potato acid phosphatase hydrolysis is between pH 4.0 and 5.0. However, many phosphoprotein substrates may be irreversibly inactivated at low pH, so incubation at pH 6.0 is recommended.

3. Add 5 to 10 U potato acid phosphatase and incubate 15 min at 30°C.

   If it is not possible to incorporate [^{32}P]phosphate into the substrate protein by metabolic labeling, control reactions should include a general phosphatase inhibitor—e.g., sodium vanadate, sodium pyrophosphate, sodium glycerophosphate, sodium orthophosphate, or p-nitrophenyl phosphate (PNPP) at a final concentration ≥10 mM—to distinguish the effects produced by protein dephosphorylation by potato acid phosphatase from those produced by contaminating proteases.

   One unit of acid phosphatase hydrolyzes 1 nmol PNPP in 1 min at 30°C and pH 4.0.

4. Remove 10 µl of the sample and add an equal volume of 2× SDS-PAGE sample buffer. Heat 5 min at 90°C.

5. Electrophorese control and dephosphorylated material on an SDS polyacrylamide gel (*UNIT 10.1*). For a radiolabeled sample, analyze the loss of radiolabel in the substrate protein by autoradiography or phosphoimage analysis; for unlabeled sample, analyze the change in substrate mobility by immunoblot analysis.

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6. Add 10 µl of 100 mM sodium pyrophosphate (10 mM final concentration) to the remaining dephosphorylated material to terminate the dephosphorylation reaction. Use an appropriate assay of protein function to characterize the changes that accompany substrate dephosphorylation.

DIGESTION OF PHOSPHOPROTEINS WITH NONSPECIFIC ALKALINE PHOSPHATASE

ALTERNATE PROTOCOL 1

Calf intestine alkaline phosphatase has been widely utilized to dephosphorylate both proteins and nucleic acids. With the widespread use of this enzyme in molecular biology most commercial preparations are essentially free of contaminating proteases. Alkaline phosphatase has a broad pH profile with an optimum between pH 8.0 and 8.5. In contrast to acid phosphatases, this enzyme can be utilized for in vitro dephosphorylation of proteins under conditions that do not denature the substrate proteins. Calf intestine alkaline phosphatase effectively dephosphorylates proteins containing phosphoserine and phosphothreonine residues, which together account for >97% of the protein-bound phosphate in eukaryotic cells. Under defined conditions, preferential dephosphorylation of phosphotyrosines by alkaline phosphatases has also been reported (Swarup et al., 1981). Alkaline phosphatase activity is stimulated by Mg^{2+} ions, although significant activity is seen even in the absence of divalent cations.

Additional Materials (also see Basic Protocol 1)

Tris/$MgCl_2$, pH 7.5 or HEPES/$MgCl_2$, pH 7.5 buffer: 50 mM Tris·Cl (pH 7.5; *APPENDIX 2E*)/1 mM $MgCl_2$ or 50 mM *N*,-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)/1 mM $MgCl_2$
Calf intestine alkaline phosphatase (molecular biology grade)

1. Incubate the sample (containing 100 to 200 µg total protein in 100 µl Tris/$MgCl_2$ or HEPES/$MgCl_2$) for 10 min at 30°C.
2. Add 20 to 30 U calf intestine alkaline phosphatase and incubate 15 min at 30°C.

   One unit of calf intestine alkaline phosphatase hydrolyzes 1 nmol p-nitrophenyl phosphate (PNPP) in 1 min at 30°C and pH 8.5.

3. Terminate the dephosphorylation reaction by adding an equal volume of 2× SDS-PAGE sample buffer or a general phosphatase inhibitor. Analyze changes in substrate mobility and function (see Basic Protocol 1).

DIGESTION OF PHOSPHOPROTEINS WITH PROTEIN SERINE/THREONINE PHOSPHATASES

BASIC PROTOCOL 2

Protein phosphatase 1, 2A, and 2B (PP1, PP2A, and PP2B, respectively) represent three major classes of protein serine/threonine phosphatases found in eukaryotic cells. These enzymes are currently available from several suppliers; they will retain full enzymatic activity for many months when stored below –20°C (see supplier's recommendations). This protocol describes the use of PP2A, a broad-specificity phosphatase, to dephosphorylate the phosphoproteins.

Materials

Sample containing 100 µg total protein
Tris/DTT/$MnCl_2$ buffer, pH 7.5: 50 mM Tris·Cl (pH 7.5; *APPENDIX 2E*)/1 mM dithiothreitol (DTT)/1 mM $MnCl_2$ (prepare fresh)
Microcystin-LR
Protein phosphatase 2A (PP2A), catalytic subunit

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1. Prepare the sample (containing 100 µg total protein) in 100 µl Tris/DTT/$MnCl_2$ buffer, pH 7.5. Prepare a control reaction in the same buffer with 1 µM microcystin-LR. Incubate 10 min at 37°C.

   Microcystin-LR is a potent inhibitor of PP1 and PP2A.

2. Add 0.2 to 0.5 U PP2A and incubate 10 to 30 min at 37°C.

   One unit of PP2A or PP1 activity hydrolyzes 1 nmol phosphorylase A in 1 min at 30°C and pH 7.5.

3. Add microcystin-LR to 1 µM final concentration to terminate the dephosphorylation reaction.

   Unlike the general phosphatases discussed above, protein phosphatases show some selectivity for the dephosphorylation of different sites in substrate phosphoproteins and can be used to gain insight into the functional role of individual protein-bound phosphates.

4. Analyze the dephosphorylated material by SDS-PAGE or functional assays (see Basic Protocol 1).

ALTERNATE PROTOCOL 2

DIGESTION OF PHOSPHOPROTEINS WITH PROTEIN TYROSINE PHOSPHATASES

Transmembrane tyrosine phosphatases such as CD45 possess low in vitro activity against many substrates, leading to the suggestion that association with extracellular ligands activates these receptor phosphatases. In this regard, protein tyrosine phosphatase 1B (PTP-1B) and src homology-protein tyrosine phosphatases (SH-PTP1 or SH-PTP2) are highly active phosphotyrosine phosphatases that demonstrate no measurable activity against phosphoserine and phosphothreonine and may be the preferred reagents to investigate the regulatory role of tyrosine phosphorylation.

Materials

Sample containing 10 to 100 µg total protein
50 mM imidazole, pH 7.5
Protein tyrosine phosphatase (e.g., PTP-1B or SH-PTP)
2× SDS-PAGE sample buffer (see Basic Protocol 1) *or* 100 mM sodium vanadate

1. Incubate the sample (containing 10 to 100 µg total protein) in 50 mM imidazole, pH 7.5, for 10 min at 37°C.

2. Add 1 to 5 U protein tyrosine phosphatase and incubate 15 to 30 min at 37°C.

   One unit of phosphatase activity hydrolyzes 1 nmol p-nitrophenyl phosphate (PNPP) in 1 min at 37°C and pH 7.0.

3. Terminate the dephosphorylation reaction by adding an equal volume of 2× SDS-PAGE sample buffer *or* 100 mM sodium vanadate to a final concentration of 0.1 mM.

   Recombinant PTP-1B and SH-PTP1 are also available as GST fusion proteins that can be bound to glutathione-agarose and conveniently removed by centrifugation or filtration following dephosphorylation of the substrate.

4. Analyze digested material by gel electrophoresis, immunoblotting, or a functional assay (see Basic Protocol 1, steps 5 and 6).

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MEASUREMENT AND IDENTIFICATION OF RELEASED ^{32}P

Time-dependent dephosphorylation of radiolabeled proteins is usually monitored by precipitating the protein substrate with 10% to 15% (w/v) trichloroacetic acid (TCA) at 4°C. Following removal of the TCA-denatured phosphoprotein by centrifugation, radioactivity that accumulates in the TCA-soluble fraction provides a monitor of [^{32}P]phosphate release. However, this procedure cannot distinguish between the release of orthophosphate and TCA-soluble phosphopeptides. To define a dephosphorylation event, the TCA-soluble radioactivity must be characterized as $^{32}P_i$. This procedure takes advantage of the unique ability of $^{32}P_i$ orthophosphate to form a complex with ammonium molybdate. The [^{32}P]phosphomolybdate complex is soluble in organic solvent and can be readily separated from ^{32}P-labeled phosphopeptides. Therefore, this assay specifically monitors protein dephosphorylation (Shenolikar and Ingebritsen, 1984).

Materials

Trichloroacetic acid
Radiolabeled protein/phosphatase reaction mixture (see Basic Protocol 1)
1.25 mM potassium phosphate (KH_2PO_4)/1 N H_2SO_4
1:1 (v/v) isobutanol/toluene
5% (w/v) ammonium molybdate
Scintillation fluid
Liquid scintillation counter

1. Add trichloroacetic acid (TCA) to dephosphorylated radiolabeled protein to give a final concentration of 10% to 15% (w/v). Incubate 10 min at 4°C.
2. Microcentrifuge 5 min at maximum speed. Remove 0.1 ml of the TCA supernatant and place in a microcentrifuge tube. Add 0.2 ml of 1.25 mM KH_2PO_4/1 N H_2SO_4 and mix.
3. Add 0.5 ml of 1:1 isobutanol/toluene to the microcentrifuge tube and mix thoroughly.
4. Add 0.1 ml of 5% ammonium molybdate and mix.
5. Microcentrifuge 5 min at maximum speed to separate aqueous and organic phases.
6. Remove 0.3 ml of the upper (organic phase), mix with 1 ml scintillation fluid, and count in a liquid scintillation counter.

COMMENTARY

Background Information

A key feature of protein phosphorylation (as compared to other protein modifications, such as proteolysis) is its reversibility. The ability to phosphorylate a substrate in vitro requires that the kinase responsible for phosphorylation be known. However, many cellular protein kinases are themselves activated via protein phosphorylation, making it difficult to use them in vitro to phosphorylate the substrate. Establishing the physiological relevance of many in vitro phosphorylations is also made difficult because these studies often do not take account of the physiological concentrations of the kinase and its substrate. In vitro experiments also tend to ignore the importance of the subcellular localization of the substrate and the kinase, sometimes leading to modifications that do not occur in vivo. For these reasons, knowledge of the physiological stimuli that regulate the substrate's function can be used to promote substrate phosphorylation as well as to provide important clues in the identification of protein kinases that phosphorylate the substrate.

Most cellular phosphoproteins are subject to covalent modification at multiple sites by more than one protein kinase. Therefore, in vitro studies with purified protein kinases often fail to recreate the spectrum of phosphorylations seen in the intact cell, and this also makes it difficult to define the role of individual co-

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valent modifications. Thus, a new strategy of analyzing the role of multisite phosphorylation has been developed using proteins that have been modified in the intact cell. However, it is particularly important to isolate such substrates in the presence of phosphatase inhibitors and to preserve the protein-bound phosphates during lengthy purification procedures.

Biochemical studies of protein phosphatases have established the in vitro substrate specificity of these enzymes, which distinguish between different phosphoamino acids (phosphoserine/phosphothreonine versus phosphotyrosine) and different phosphoprotein substrates. These enzymes can also show preferential dephosphorylation of specific sites (Shenolikar and Nairn, 1991; Charbonneau and Tonks, 1992; Shenolikar, 1994). In vitro dephosphorylation by selected phosphatases may not only define the functional role of selected covalent modifications but also identify "silent" or "structural" phosphorylations that do not directly regulate the protein function. These phosphorylations may play an indirect role in modulating protein phosphorylation or dephosphorylation of other sites.

This unit has focused on commercially available phosphatases that have been used to characterize the functional importance of protein phosphorylation. For example, several growth-associated kinases, including the mitogen-activated protein kinases (MAP kinases) and the cyclin-dependent kinases such as cdc2, are phosphorylated on adjacent threonine and tyrosine residues. Phosphorylation of these enzymes is controlled by kinases and phosphatases that are themselves highly regulated in the intact cell. In vitro dephosphorylation by serine/threonine-specific or tyrosine-specific phosphatases both established the functional importance for phosphorylations even prior to the identification of the kinases and phosphatases that regulate these proteins.

The most direct method for detecting protein phosphorylation is clearly metabolic labeling of cells with $^{32}P_i$ (see *UNIT 13.2*) and subsequent isolation of the ^{32}P-labeled substrate protein. However, it is often difficult to develop a rapid purification procedure for the specific protein of interest without selective reagents such as monospecific antibodies or other affinity ligands. Significant labeling of proteins is also a problem given the low abundance of many regulatory proteins and the slow turnover of their protein-bound phosphate under "basal" conditions. In cases where [^{32}P]phosphate can be readily incorporated into the protein, in vitro dephosphorylation by selected phosphatases can establish the functional importance of the modification. Ability to correlate loss of [^{32}P]phosphate from a specific polypeptide with a functional change provides the best evidence for the physiological importance of protein phosphorylation.

Initial experiments should use a general phosphatase such as potato acid phosphatase or calf intestine alkaline phosphatase which can successfully dephosphorylate a wide variety of phosphoproteins modified on several different amino acids. It should be noted that acid phosphatases from animal tissues (e.g., bone, prostate, liver, and heart) represent a class of low-molecular-weight (18,000- to 25,000-kDa) protein tyrosine phosphatases that selectively dephosphorylate phosphotyrosine residues. These enzymes were defined as acid phosphatases because they hydrolyzed *p*-nitrophenyl phosphate (PNPP) at a pH optimum between 4.0 and 5.0. However, at physiological pH, these enzymes appear to specifically dephosphorylate proteins and peptides containing phosphotyrosine residues. The mammalian acid phosphatases show no significant activity against phosphoserine/phosphothreonine-containing proteins.

With phosphoprotein substrates, potato acid phosphatase shows a preference for dephosphorylating phosphotyrosine residues, followed by phosphothreonine residues. By comparison, this enzyme shows very low activity against phosphoserine-containing substrates. Wheat germ acid phosphatase can dephosphorylate phosphoserines but still shows significantly higher activity against phosphotyrosine and phosphothreonine (Van Etten and Waymack, 1991). In this regard, alkaline phosphatase is often preferred over potato or wheat germ acid phosphatase for dephosphorylation of phosphoserine, the predominant protein modification in eukaryotic cells.

When a general phosphatase, such as alkaline phosphatase, is used, effects independent of protein dephosphorylation must be considered. For instance, alkaline phosphatase hydrolyses ATP and inhibits ATP-dependent processes, such as protein kinases and ATP-dependent ion channels that are also inhibited by protein dephosphorylation (Berger et al., 1993). To distinguish between these differing effects, use of immobilized alkaline phosphatase is recommended. Agarose or dextran beads covalently linked to alkaline phosphatase should be washed extensively with the assay buffer to remove storage solutions and any

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unbound alkaline phosphatase. Following incubation with the substrate protein, immobilized alkaline phosphatase can be removed by centrifugation or filtration. Functional analysis of the substrate can then be undertaken without concern for the potential effects of the phosphatase on such analysis. However, if the substrate is present in a particulate fraction (e.g., in subcellular organelles), it cannot be easily separated from the beads by centrifugation or filtration. In that case, the phosphatase should be inhibited with a general inhibitor such as sodium pyrophosphate. In contrast to the general phosphatases, protein phosphatases are inefficient at hydrolyzing ATP and do not hinder analysis of ATP-dependent processes.

Once phosphorylation of a substrate is established using a general phosphatase, the next step is to use protein phosphatases that have much higher activities against phosphoprotein substrates than either acid or alkaline phosphatase and are highly selective for specific phosphoamino acids. Protein phosphatases 1, 2A, and 2B (PP1, PP2A, and PP2B) represent three major classes of protein serine/threonine phosphatases in eukaryotic cells. These enzymes differ in their subunit structure, substrate specificity, and regulation by divalent cations. PP1 and PP2A account for >90% of the total protein serine/threonine phosphatase activity in many cell extracts (Shenolikar and Nairn, 1991) and demonstrate a broad in vitro substrate specificity. Therefore, PP1 and PP2A are the most commonly used reagents for in vitro dephosphorylation of phosphoserine- and phosphothreonine-containing proteins. PP1 and PP2A are inhibited by microcystin-LR as well as several other recently discovered inhibitors, including okadaic acid, calyculin A, and tautomycin, albeit with differing IC_{50} values.

Inhibitors, such as okadaic acid (with IC_{50} for PP2A of 0.1 to 1.0 nM and for PP1 of 10 to 100 nM), calyculin A (with an IC_{50} for PP1 of 1 nM and for PP2A of 10 nM), and microcystin-LR (with an IC_{50} for both PP1 and PP2A of 0.1 nM) are used to exclude nonspecific effects of PP1 and PP2A. These inhibitors represent important experimental tools for establishing the physiological importance of reversible protein phosphorylation.

PP2B, also called calcineurin, is a Ca^{2+}/calmodulin-dependent phosphatase and demonstrates a narrow in vitro substrate specificity compared to PP1 or PP2A. The enzyme purified from mammalian tissues consists of a catalytic subunit that is tightly associated with a calcium-binding regulatory subunit. PP2B has no activity in the absence of 1 μM calmodulin and 1 mM $CaCl_2$ and can be activated by either Ca^{2+}/calmodulin or 1 mM $MnCl_2$. Many highly purified PP2B preparations show no activity in the presence of Ca^{2+}/calmodulin but are fully activated by Mn^{2+}. The immunosuppressive drugs cyclosporin A and FK506 selectively inhibit PP2B in vivo and in vitro.

PP2B in the presence of Ni^{2+} ions and PP2A in association with an endogenous regulator protein or viral antigens can dephosphorylate proteins and peptides containing phosphotyrosines. However, under the conditions described in Basic Protocol 2, PP1, PP2A, and PP2B are highly selective for dephosphorylation of phosphoserine and phosphothreonine residues. PP1 and PP2A represent the major phosphohistidine phosphatase activity measured in tissue extracts, but the regulatory importance of this modification has not yet been established.

Critical Parameters

Some commercial preparations of potato acid phosphatase contain contaminating protease activity. Because phosphorylation sites reside near the surface of the substrate protein, they are often accessible to proteases, so functional changes that result from limited proteolysis can be incorrectly attributed to dephosphorylation. To limit the effects of proteases, high enzyme concentrations or prolonged incubations with the general phosphatases should be avoided. Aside from including protease inhibitors in the reaction, two strategies should be considered to distinguish proteolysis from dephosphorylation. Formation of a complex between trichloroacetic acid–soluble [^{32}P]phosphate and ammonium molybdate (Support Protocol) is particularly useful in distinguishing [^{32}P]phosphate from ^{32}P-labeled phosphopeptides. The use of phosphatase inhibitors in control reactions also identifies those effects that can be attributed to reactions in which there are contaminating proteolytic enzymes.

Commercial preparations of PP2A and PP1 contain only the free catalytic subunits. In vivo these subunits associate with regulatory subunits, which modulate their substrate specificity. However, the catalytic subunits are the most readily purified to homogeneity and can be stored for prolonged periods without significant changes in their enzymatic properties. Both PP1 and PP2A catalytic subunits retain high activity in the absence of divalent cations; PP2A activity may be further stimulated by 1

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mM Mn^{2+} in the enzyme reaction. Long-term storage of PP1 and PP2A results in an apparent loss of enzyme activity. However, these inactive enzymes can be fully reactivated by including 1 mM $MnCl_2$ in the assay. The Mn^{2+}-dependent PP1 enzyme shows slightly altered substrate specificity when compared with PP1 purified from tissues. Moreover, PP1 is potentially inhibited by several endogenous inhibitors. Thus, PP2A may be the preferred reagent for in vitro dephosphorylation of substrates in many cell extracts.

Troubleshooting

The absence of a functional change following incubation of the substrate with one or more phosphatases may indicate either that the protein was not phosphorylated or that the dephosphorylation of existing phosphates has no effect on protein function. It is also possible that components in the reaction mixture inhibited the phosphatases. The latter possibility can be readily established if the substrate protein has been metabolically labeled with [^{32}P]phosphate. SDS-PAGE followed by autoradiography or phosphoimage analysis can directly monitor the time-dependent loss of radiolabel from substrate protein. Coincidence between the removal of radiolabeled phosphate and change of function of the substrate protein can be used to distinguish between important regulatory modifications and "silent" phosphorylations. In analyses of unlabeled substrates, the reaction should include an internal standard or a known ^{32}P-labeled protein or peptide. Dephosphorylation of the radiolabeled marker in the presence or absence of the sample should establish the extent to which activity of the added phosphatase has been compromised by components in the sample. If several different phosphatases fail to elicit a change in protein function under conditions that dephosphorylate the marker, the substrate protein may contain no protein-bound phosphate or phosphorylation may not be functionally important for the protein.

Anticipated Results

Most phosphoproteins should be dephosphorylated by the general phosphatases, leading to complete removal of protein-bound phosphate and functional changes indicating that reversible phosphorylation controls the protein function. In a number of cases, metabolic labeling and phosphoamino acid analysis or the use of specific protein (phosphoserine/phosphothreonine or phosphotyrosine) phosphatases has successfully identified the amino acids modified. Such studies have then located the phosphorylated residues, especially in proteins whose primary structures have been determined by amino acid sequencing or cDNA cloning. A combination of biochemical studies and site-directed mutagenesis has then been used to establish the functional role of phosphorylation at individual sites. Increasing evidence points to the presence of both positive and negative regulatory phosphorylations in proteins. Thus, it is difficult to predict the functional outcome of complete in vitro dephosphorylation of many substrates.

Time Considerations

Approximately 1 hr is required to complete the dephosphorylation reaction. SDS-PAGE and analysis of the gel require 1.5 hr. The time required for functional analysis depends on the particular assay used.

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Production of Antibodies That Recognize Specific Tyrosine-Phosphorylated Peptides

It is possible to produce anti-phosphopeptide antibodies (i.e., antibodies recognizing phosphorylated peptides) that recognize a protein only in its phosphorylated state, and that do not cross-react with either the cognate unphosphorylated protein or other phosphoproteins. Because the state of phosphorylation is often indicative of a protein's functional state or activity, such antibodies provide a convenient probe of the functional state of a protein. Thus, unlike conventional antibodies, anti-phosphopeptide antibodies provide information regarding not only the abundance of a protein but also its activity. Unlike general anti–phosphoamino acid (e.g., anti-phosphotyrosine) antibodies, which have broad reactivity, anti-phosphopeptide antibodies may have unique specificity toward the cognate proteins. Such reagents not only facilitate conventional in vitro analysis of phosphoproteins, but also allow heretofore impossible applications—e.g., differential isolation of species of a particular protein that have been phosphorylated at individual phosphorylation sites, as well as analysis of the functional state of a protein in situ by immunohistochemical techniques.

The Basic Protocol describes the production of polyclonal anti-phosphopeptide antibodies. This procedure is based upon immunizing animals with oligopeptides that have been synthesized containing phosphorylated tyrosine; hence, a knowledge of the sequence and phosphorylation site of the protein of interest is a prerequisite. Such an immunization will generate an immune response with at least four components: (1) anti–carrier protein reactivity, (2) general anti-phosphotyrosine reactivity, (3) phosphorylation-independent anti-peptide reactivity, and (4) phosphorylation-dependent anti-peptide reactivity. A multiple-step affinity chromatographic purification with several negative-selection steps is carried out to produce a final antibody preparation having the desired reactivity. Support Protocols are provided for peptide synthesis (Support Protocol 1) and for the coupling of peptides (Support Protocol 2) and phosphotyrosine (Support Protocol 3) to the affinity matrix (Affi-Gel 10); BSA-agarose affinity matrix is commercially available. General methods for the coupling of peptides to carrier proteins for use in immunization are described in Collawn and Paterson (1991).

This unit describes production of antibodies against tyrosine-phosphorylated peptides, with which the authors have the most expertise, but the principles discussed here also apply to peptides phosphorylated on serine and threonine (see Key References).

STRATEGIC PLANNING

The first step of the planning phase is design and synthesis of the required peptides (see Support Protocol 1 and Background Information). The authors employ a peptide containing fifteen amino acids with the phosphorylation site Tyr in the center (residue 8). This peptide consists of fourteen amino acids from the actual sequence of the protein plus an N-terminal Lys to enhance coupling of the peptide to a carrier protein (BSA is used as a carrier in this protocol). The peptide, coupled to BSA, is then used to immunize rabbits. The cognate phosphopeptide is also immobilized on an affinity-chromatography column support (see Support Protocol 2); this affinity matrix will ultimately be used to purify the antibody from the rabbit serum. In addition, a series of negative-selection affinity-chromatography columns are used to adsorb antibodies from the serum that cross-react with epitopes other than the cognate phosphopeptide. These columns are composed of matrix coupled to various synthetic peptides—principally the cognate nonphosphorylated peptide (to remove phosphorylation-independent antibodies) and phosphotyrosine itself (to

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remove indiscriminant anti-phosphotyrosine reactivity; see Support Protocol 3 for preparation of phosphotyrosine affinity columns). Additional negative-selection steps may be used if needed, depending upon the particular target chosen, to remove antibodies that cross-react with phosphorylation sites of other proteins having known homology to the target site. This cross-reactivity occurs particularly when the target protein is a member of a family of homologous proteins—e.g., a subgroup of related receptor tyrosine protein kinases. If such cross-reactivity is anticipated, it is possible to synthesize not only the cognate phosphorylated and nonphosphorylated peptides, but also phosphopeptides based upon the related homologous sequences, to be used for negative selection. The authors also pass the serum over a column consisting of immobilized carrier protein (commercially available BSA-agarose) to remove the majority of the antibodies generated against the much larger carrier protein. Thus, a typical purification scheme might consist of the following:

Negative-selection affinity-purification columns

- BSA (carrier)
- phosphotyrosine
- cognate nonphosphopeptide
- homologous phosphopeptides (if desired)

Positive-selection affinity-purification column

- cognate phosphopeptide.

The second step in planning is to raise and test antisera in rabbits. To prepare the phosphopeptide-carrier conjugate for immunization of rabbits, the authors couple a 30-fold molar excess of phosphopeptide (14.4 mg dissolved in water and neutralized with 1 N NaOH) to BSA (20 mg dissolved in 0.4 M sodium phosphate buffer, pH 7.5), using glutaraldehyde as the cross-linking reagent as described in Doolittle (1986). The reaction is allowed to proceed 30 min at room temperature; the development of a yellow color in the coupling solution is an indication that the glutaraldehyde has reacted. General methods for coupling of peptides to carrier proteins for use in immunization are described in Collawn and Paterson (1991). Conjugation can be confirmed by performing an anti-phosphotyrosine immunoblot (*UNIT 13.4*) on a small test aliquot of the conjugate (although a smear should be expected on the blot, resulting from variation in the amount of peptide coupled per BSA molecule as well as from possible multimers of BSA). Guidelines for immunization of rabbits are provided in Cooper and Paterson (1989), and many institutions have core facilities for the immunization of rabbits and production of antisera. The authors immunize with 1 mg of immunogen (the peptide-BSA conjugate prepared above, dissolved in PBS) in 1 ml of a 50% emulsion with complete Freunds adjuvant. This is administered subcutaneously at multiple sites, followed by four boosts at 2-week intervals using same quantity of immunogen in incomplete Freunds adjuvant. An adequate immune response can be seen as early as week 6.

The specificity of the final antibody is demonstrated most simply by immunoblot analysis (*UNIT 10.10*) using a panel of relevant phosphorylated and nonphosphorylated proteins. Electrophoresis is carried out with purified cognate protein in both its phosphorylated and unphosphorylated states, as well as with any proteins toward which there could conceivably be cross-reactivity. These are most easily isolated by immunoprecipitation using conventional antibodies. The blot is probed with the purified anti-phosphopeptide antibody to demonstrate appropriate reactivity. This immunoblot assay may also be used after each chromatographic step to monitor the success of the purification.

An assay also must be devised for estimating the titer of the immune response in the sera of the immunized rabbits. Convenient screening assays for this purpose include tests for

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the ability of crude sera to immunoprecipitate the target phosphoprotein from a cell lysate known to contain it, as well as immunoblotting of purified phosphorylated target protein with the crude serum.

The relationship among the above procedures is illustrated by the flow chart in Figure 13.6.1.

PRODUCTION OF POLYCLONAL ANTI-PHOSPHOPEPTIDE ANTIBODIES

BASIC PROTOCOL

The purification of polyclonal antibodies consists of multiple affinity-chromatography steps for the negative and positive selection of antibodies of the appropriate reactivity (see Fig. 13.6.1). All of the following chromatographic steps may be carried out at room temperature, with the solutions applied to the columns by gravity.

Materials

BSA-agarose affinity matrix (Sigma) packed (as in Support Protocol 2) in a 10-ml bed volume column

Phosphotyrosine affinity matrix column (10-ml bed volume; see Support Protocol 3)

Crude serum from rabbit immunized with phosphopeptide-BSA conjugate (see Strategic Planning)

PBS/azide: PBS (*APPENDIX 2E*) containing 0.02% (w/v) sodium azide (store indefinitely at 4°C or room temperature)

Cognate nonphosphopeptide affinity matrix column (3-ml bed volume; see Support Protocols 1 and 2 and see Strategic Planning)

3 M NaSCN

Homologous phosphopeptide affinity matrix columns (optional; 3-ml bed volume; see Support Protocols 1 and 2 and see Strategic Planning)

Positive-selection phosphopeptide affinity matrix column (3-ml bed volume; see Support Protocols 1 and 2 and see Strategic Planning)

3.5 M and 4.5 M $MgCl_2$ (optional; see Critical Parameters)

Spectrophotometer (optional)

Dialysis tubing (MWCO 12,000 to 14,000; 10 mm width, 6.4 mm diameter; e.g., Spectra/Por 4 from Spectrum)

Additional reagents and equipment for dialysis (*UNIT 4.4 & APPENDIX 3B*), protein quantitation (*UNIT 3.4*), and analysis of antibodies by immunoblotting (*UNIT 10.10*)

Deplete reactivity of serum toward phosphotyrosine and carrier protein

1. Connect the washed BSA-agarose and phosphotyrosine affinity matrix columns in series.

2. Pass ~15 ml crude serum through the two columns, allowing it to flow through by gravity. Wash with PBS/azide until all of the yellow color of the serum has passed through the columns (or monitor the A_{280} of the column effluent spectrophotometrically until baseline absorbance is reached), then wash with an additional 5 to 10 ml PBS/azide, collecting all washings in the flowthrough fraction, which will contain the antibody of interest.

   Aliquots of the crude serum as well as this first flowthrough may be saved for subsequent analysis and comparison of individual purification fractions. The serum may be analyzed at this time for elimination of anti-phosphotyrosine reactivity by performing immunoblotting (UNIT 10.10) of samples containing a variety of phosphotyrosyl proteins; alternatively it is possible to save an aliquot for analysis and proceed to the next step (see Troubleshooting).

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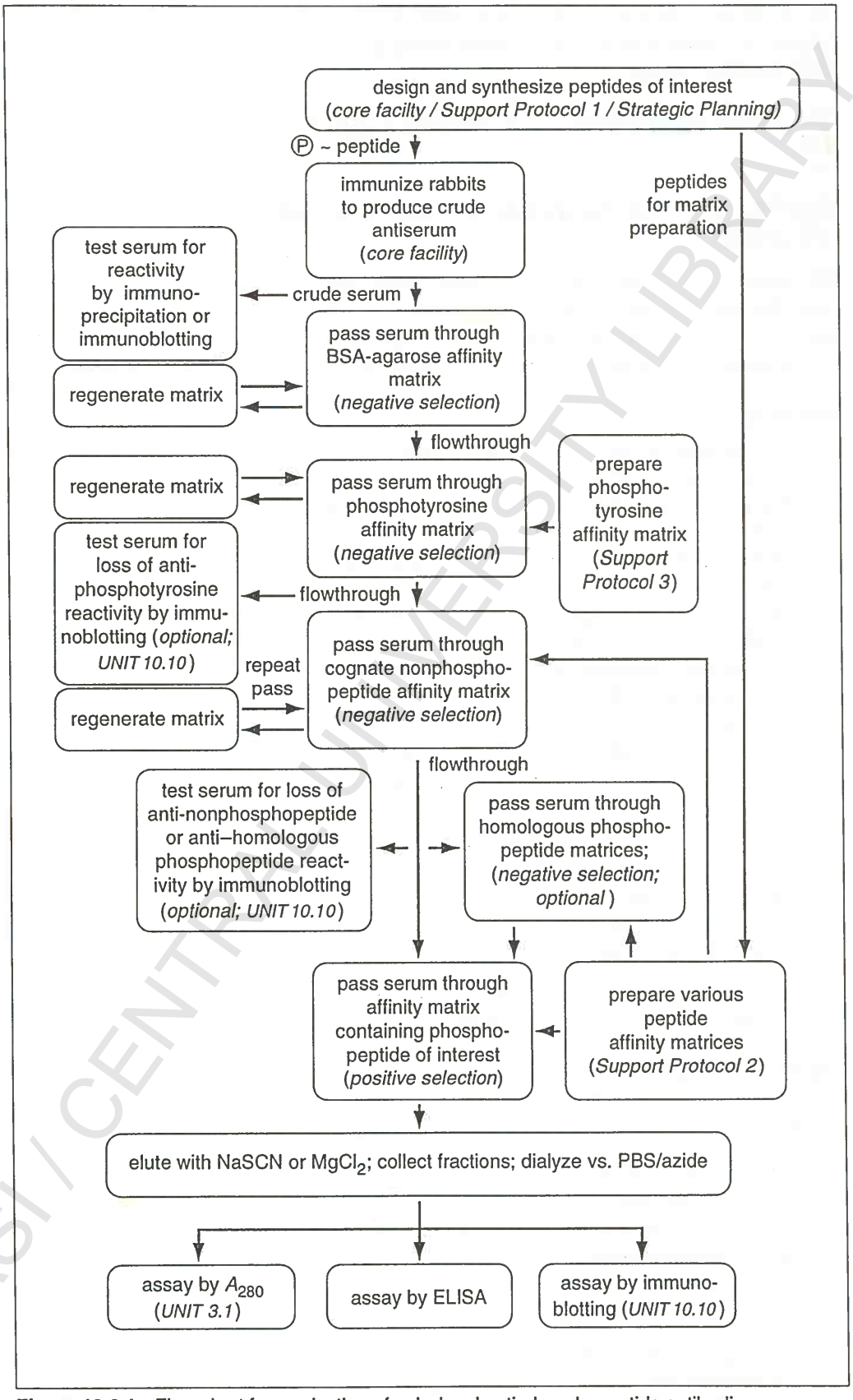

Figure 13.6.1 Flow chart for production of polyclonal anti-phosphopeptide antibodies.

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Expect that with each passage through a column, the volume of the serum will increase, which will prolong the amount of time needed for subsequent columns. Note that if flowthrough is monitored by the passage of the yellow color of the serum, this will become more difficult as the serum becomes more dilute.

After the serum passes through, regenerate the columns by washing with 10 bed volumes of 3 M NaSCN followed by 10 bed volumes of PBS/azide, and store at 4°C in PBS/azide for future use.

Deplete reactivity of serum toward cognate nonphosphopeptide

3. Pass the flowthrough serum through the nonphosphopeptide affinity matrix column by gravity as many times as necessary to deplete cross-reactivity, regenerating the column between passes by washing with 10 bed volumes of 3 M NaSCN followed by 10 bed volumes PBS/azide.

   A 15-ml starting serum sample may have to be passed over this column several times to quantitatively deplete cross-reactivity (see Critical Parameters). The serum may be analyzed after each or several passes, or an aliquot can be saved for analysis at a later time.

4. If cross-reactivity with homologous phosphoprotein(s) is anticipated, pass the flowthrough serum through the homologous phosphopeptide affinity matrix column(s) using the methodology described in step 3.

   See Strategic Planning for discussion of cross-reactivity with homologous phosphoproteins.

Purify antibodies by positive-selection affinity chromatography and dialysis

5. Hydrate and wash ~25-cm strips of dialysis tubing in advance for collection of fractions from the positive-selection affinity purification. Secure one end of each length of tubing with a dialysis clamp and check for leaks (*UNIT 4.4 & APPENDIX 3B*). Also prepare 6 liters PBS/azide and cool to 4°C in advance to use as the dialysis solution.

   To minimize the time that the antibodies are exposed to the elution solution, fractions (~3 ml each) from the positive-selection affinity column will be collected directly into prepared dialysis tubing.

6. Pass the flowthrough serum from the previous chromatography step through the positive-selection phosphopeptide affinity column three times, without washing between passes.

   Three passes are performed to maximize the interaction of the antibody with the affinity matrix.

7. Collect the flowthrough serum from the final pass, then wash the column with 5 to 20 ml PBS/azide (depending upon the precolumn volume and column bed volume) and combine the washings with the flowthrough serum. Wash with an additional 20 ml PBS/azide, and collect the washings separately as the "wash" fraction.

8. Elute with chaotropic agent of choice: either 20 ml of 3 M NaSCN, or 10 ml of 3.5 M $MgCl_2$ followed by 10 ml of 4.5 M $MgCl_2$. Upon starting the elution, immediately begin collecting ~3-ml fractions directly into the dialysis bags prepared in step 5, securing the proximal end of the tubing with dialysis clamps and dropping the bag immediately into the PBS/azide dialysis solution. Collect at least six fractions.

   Alternatively, ~3-ml fractions can be collected in tubes and the liquid immediately placed into dialysis tubing upon completion of each fraction. Most of the antibody will elute in the first three fractions from a column of 2- to 3-ml bed volume. Regenerate the column as in step 3.

   See Critical Parameters for a discussion of the relative merits of NaSCN versus $MgCl_2$ as eluants.

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9. Dialyze all fractions collected in step 8 exhaustively against PBS/azide at 4°C (*UNIT 4.4 & APPENDIX 3B*).

Analyze and store purified antibody

10. Determine protein concentration of dialyzed fractions by measuring absorbance at 280 nm or by a colorimetric protein assay (*UNIT 3.1*) and calculate yield.

    The authors have generally recovered ≥1 mg of purified antibody from a 15-ml serum sample.

11. Store the antibody in aliquots at –70°C; store working aliquot at 4°C.

12. Assay the reactivity and cross-reactivity of the final samples, as well as any aliquots saved from previous steps, using immunoblotting (*UNIT 10.10*).

    Alternatively, an ELISA (Hornbeck, 1991) can be used to assay the samples.

SUPPORT PROTOCOL 1

SYNTHESIS OF PEPTIDES

Many institutions have core facilities for synthesis of peptides using automated solid-phase peptide synthesizers. For phosphotyrosine-containing peptides, the authors originally used standard Merrifield solid-phase procedures with *t*-butyloxycarbonyl (Boc) amino acids and Boc-*O*-(dibenzylphosphono)-L-tyrosine as the phosphorylated residue (Barany and Merrifield, 1979). For cleavage of phosphopeptide from the resin, 1 g of phosphopeptide resin was stirred 10 min at 0°C with 2 ml dimethyl sulfide; then an ice-cold mixture of 4 ml trifluoromethanesulfonic acid (TFMSA) and 10 ml of trifluoroacetic acid was added slowly with stirring. The resulting reaction mixture was stirred under a nitrogen atmosphere 4 hr at 0°C and the cleaved phosphopeptide precipitated with methyl *t*-butyl ether at –30°C. The precipitate was dissolved in 40% acetic acid and lyophilized several times from water. This original method suffers from low yields. Currently, most phosphotyrosyl-containing peptides are synthesized with 9-fluorenylmethyloxycarbonyl (Fmoc) phosphotyrosine. The lack of a side chain protecting group on the phosphotyrosine (Kitas et al., 1994) allows standard Fmoc synthesis and cleavage procedures to be employed (Chang and Meienhofer, 1978). However, a higher molar excess of activated Fmoc amino acids than that used in standard procedures may be required for efficient coupling of amino acids after unprotected phosphotyrosine has been added. In some circumstances, a double coupling procedure may be employed to add the few amino acids following phosphotyrosine to the growing peptide chain. This alternative requires the sequential, repeated addition of the next particular amino acid to be added to the peptide. A support protocol for the coupling of peptides to an affinity matrix is provided (see Support Protocol 2); general methods for coupling peptides to carrier proteins are described in Collawn and Paterson (1991).

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COUPLING OF PEPTIDES TO AFFI-GEL 10 AFFINITY MATRIX

This protocol describes coupling of phosphopeptides or nonphosphopeptides to Affi-Gel 10 affinity matrix for use in affinity column chromatographic purification of antibodies. Anhydrous coupling, detailed here, is the most efficient coupling method for peptides. The steps here result in production of 3 ml final bed volume of affinity resin; 3 µmol of peptide is coupled per milliliter Affi-Gel 10. According to the manufacturer's specifications, Affi-Gel 10 resin contains ~15 µmol of active ester per milliliter of gel, and the gel has a capacity for 35 mg of protein or 15 to 20 µmol of a low-molecular-weight ligand per milliliter of gel. Note that these capacities are dependent upon the molecular weight and nature of the protein. After coupling, the matrix may be poured into columns of the appropriate size.

Materials

Synthetic oligopeptide for coupling (see Support Protocol 1)
Dimethyl sulfoxide (DMSO)
N-methylmorpholine (99% purity; Acros Organics)
Affi-Gel 10 (Bio-Rad) or equivalent activated support matrix
Ethanolamine
0.1 M ethanolamine·HCl, pH 8.0
High-salt/high-pH solution: 0.5 M NaCl/0.4% (w/v) sodium bicarbonate
High-salt/low-pH solution: 0.5 M NaCl/100 mM sodium acetate, pH 4.2
PBS/azide: PBS (*APPENDIX 2E*) containing 0.02% (w/v) sodium azide
0.5 M NaCl
3 M NaSCN

Polypropylene screw-cap centrifuge tubes (do not use polystyrene tubes with DMSO)
End-over-end rotator
IEC Clinical centrifuge (or equivalent)
Vacuum aspirator
Glass chromatography columns, ≥5-ml capacity (Bio-Rad)

Prepare peptide solution

1. Dissolve peptide in DMSO at a volume ~0.5 to 4 times the desired bed volume.

   For a 3-ml bed volume, dissolve a total of 9 µmol peptide, which for an average 15-mer peptide of mol. wt. ~1700 would be 15.3 mg.

2. Neutralize the peptide solution by titration as follows: add peptide synthesis–grade *N*-methylmorpholine (full-strength or diluted to 1:1 to 1:9 in DMSO) in 1 to 2 µl increments, remove a 2 to 3 µl aliquot after each addition, dilute this with 50 µl water, then spot it onto a strip of pH paper. Repeat this process until a pH in the range of 7 to 8 is reached.

   IMPORTANT NOTE: *Titrate carefully, as it is very easy to overshoot the desired pH. Typically 7 to 8 µl of N-methylmorpholine is required per 3 µmol peptide, although the precise amount will depend greatly upon the particular amino acid sequence. The aliquots must be mixed with water because pH is a measure of hydrogen ion concentration, and hence the pH of a nonaqueous solution cannot be measured.*

Add peptide to Affi-Gel 10

3. Mix contents of the bottle of Affi-Gel 10 matrix well to resuspend the resin, then transfer the required amount of resin to a screw-cap polypropylene centrifuge tube and wash three times in DMSO, each time by centrifuging 5 min at 700 × *g* (2000 rpm in an IEC Clinical centrifuge, setting 5), room temperature, aspirating the

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supernatant, resuspending the resin in 5 vol DMSO, then centrifuging again at 700 × *g*.

IMPORTANT NOTE: *Be sure to take at least twice as much Affi-Gel 10 as ultimately will be needed, as the volume will shrink. The resin is supplied as a 50% slurry; therefore, it is necessary to take at least four times the required bed volume. Avoid centrifugation at higher than recommended speed as this may result in collapse of the resin.*

4. Aspirate the excess DMSO from the resin and add the peptide solution prepared in step 2. Incubate overnight at room temperature with end-over-end rotation.

Quench and wash the resin

5. Add 2 µl pure ethanolamine per ml resin and incubate 2 hr at room temperature with end-over-end rotation.

   Ethanolamine is added directly to the coupling reaction to quench unreacted ester groups.

6. Wash twice in DMSO as in step 3.

7. Wash twice in 0.1 M ethanolamine·HCl, pH 8.0, as in step 3 (performing the first wash on ice as a great deal of heat will be generated). Remove the 0.1 M ethanolamine·HCl from the second wash and replace with fresh, incubate tube overnight at 4°C with end-over-end rotation, then centrifuge at low speed and aspirate the supernatant.

8. Wash three times with high-salt/high-pH solution using the technique described in step 3.

9. Wash three times with high-salt/low-pH solution using the technique described in step 3.

10. Wash three times with PBS/azide using the technique described in step 3 and store washed resin at 4°C until ready to pack in column.

11. Mix matrix into a slurry with 0.5 M NaCl and pour into glass chromatography columns.

    Commercially available glass chromatography columns can be used; alternatively, 5-ml plastic syringes with 1-cc plugs of silanized glass wool in the bottom may function as columns.

12. Wash each column with 10 column volumes 3 M NaSCN followed by 10 column volumes PBS/azide.

SUPPORT PROTOCOL 3

COUPLING OF PHOSPHOTYROSINE TO AFFI-GEL 10 AFFINITY MATRIX

This protocol describes coupling of phosphotyrosine to Affi-Gel 10 affinity matrix for use in affinity column chromatographic purification of antibodies. Phosphotyrosine is insoluble under the anhydrous conditions used for coupling peptides (see Support Protocol 2); thus coupling is done under aqueous conditions. The steps here result in production of 10 ml final bed volume of affinity resin; as for peptides, 3 µmol phosphotyrosine is coupled per milliliter of Affi-Gel 10. According to the manufacturer's specifications, Affi-Gel 10 resin contains ~15 µmol of active ester per ml of gel, and the gel has a capacity for 35 mg of protein per ml gel or 15 to 20 µmol of a low-molecular-weight ligand per ml gel. Note that these capacities may vary depending upon the molecular weight and nature of the protein. Phosphotyrosine-agarose affinity matrix is now also available commercially from Sigma.

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Materials

Phosphotyrosine
1 M NaOH (optional)
0.4% (w/v) sodium bicarbonate

Fritted-glass funnel and vacuum aspirator
Glass chromatography column, ≥14-ml capacity (Bio-Rad)

Additional reagents and equipment for coupling peptides to Affi-Gel 10 affinity matrix (see Support Protocol 2)

Prepare phosphotyrosine solution

1. Dissolve phosphotyrosine in 0.4% sodium bicarbonate at a volume ~0.5 to 4 times the desired bed volume.

   For a 10-ml bed volume, dissolve a total of 30 µmol phosphotyrosine. Based on its molecular weight of 261, this amounts to 7.8 mg, although it is acceptable (and may be easier) to use an excess, as phosphotyrosine is relatively inexpensive.

2. Check that the pH is in the range of 7 to 8 using pH paper. If adjustment is necessary, neutralize the solution by titration—i.e., adding small amounts of 1 M NaOH, removing a 2- to 3-µl aliquot after each addition, then spotting the aliquots onto a strip of pH paper. Repeat this process until a pH in the range of 7 to 8 is reached.

Add phosphotyrosine to Affi-Gel 10

3. Mix contents of the bottle of Affi-Gel 10 matrix well to resuspend resin, then transfer the required amount of resin to a fritted-glass funnel attached to a vacuum aspirator. Wash three times, each time by pouring cold distilled water over the resin in the funnel and aspirating, then perform one final wash in the same manner using ice-cold 0.4% sodium bicarbonate.

   IMPORTANT NOTE: *Be sure to take at least twice as much Affi-Gel 10 as ultimately will be needed, as the volume will shrink. The resin is supplied as a 50% slurry; therefore, it is necessary to take at least four times the required bed volume. Do not allow more than 20 min to elapse from the time the resin is removed from the bottle to the time it is mixed with the phosphotyrosine solution.*

4. Remove most of the excess liquid from the gel by vacuum aspiration without letting it dry completely, transfer it to a screw-cap centrifuge tube, and add the phosphotyrosine solution from step 2.

5. Incubate overnight at 4°C with end-over-end rotation.

Quench and wash resin

6. Add 2 µl pure ethanolamine per ml resin and incubate 2 hr at room temperature with end-over-end rotation.

   Ethanolamine is added directly to the coupling reaction to quench unreacted ester groups.

7. Wash resin twice, each time by centrifuging at low speed (see Support Protocol 2, step 3), aspirating the supernatant, resuspending the resin in 5 vol of 0.4% sodium bicarbonate, then centrifuging again at low speed.

8. Wash resin twice with 0.1 M ethanolamine·HCl, pH 8.0, as in step 7. Remove the 0.1 M ethanolamine from the second wash and replace with fresh, incubate tube overnight at 4°C with end-over-end rotation, then centrifuge at low speed and aspirate the supernatant.

9. Wash, store, and pack resin in columns (see Support Protocol 2, steps 8 to 12).

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COMMENTARY

Background Information

Conventional antibodies have proven to be invaluable tools in numerous techniques for the biochemical analysis of proteins. In the past, antibodies with specificity toward the phosphorylated forms of proteins were produced serendipitously. Using techniques such as those described in this unit, such antibodies may now be produced by design. Whereas phosphorylated holoproteins might in general be poor candidates for immunogens in the production of such antibodies as a result of their susceptibility to dephosphorylation, it has been suggested that short phosphopeptides are relatively resistant to phosphatases (Czernik et al., 1991), thereby providing a better chance of success.

A dramatic advance in the analysis of protein tyrosine phosphorylation and the regulation of signal transduction pathways by such phosphorylation occurred with the production of polyclonal and monoclonal anti-phosphotyrosine antibodies (Frackelton et al., 1983; Ross et al., 1981; reviewed in Stern, 1991). These antibodies proved capable of recognizing phosphorylated tyrosine residues in the context of virtually any flanking peptide sequence, and were shown to be exquisitely specific in the requirement for a phosphorylated tyrosine yet at the same time remarkably promiscuous in their acceptance of any peptide sequence. Techniques using polyclonal and monoclonal anti-phosphotyrosine antibodies have supplanted the standard methodology of metabolically labeling proteins with $^{32}P_i$, which was cumbersome, time-consuming, hazardous, and of relatively lower sensitivity.

Antibodies that recognize a specific tyrosyl-phosphorylated peptide as described in this unit represent a marriage of anti-phosphotyrosine technology with the stringent sequence-dependent specificity of conventional antibodies (Bangalore et al., 1992; DiGiovanna and Stern, 1995; Epstein et al., 1992; Roussel, 1990). Conceptually, they may be thought of either as anti-phosphotyrosine antibodies that have strict sequence specificity, or as conventional antibodies possessing the additional specificity of phosphorylation dependence.

Anti-phosphotyrosine antibodies have been most useful in the analysis of tyrosine phosphorylation of proteins in a technique using a combination of immunoprecipitation and immunoblotting (known as the "IP-western" method). In this procedure, a protein typically is immunoprecipitated either with conventional anti-protein antibody or with anti-phosphotyrosine antibody, then immunoblotted with whichever of these two antibodies was not used for the immunoprecipitation. Because anti-phosphopeptide antibodies recognize the cognate protein, but only when it is phosphorylated, they are capable of performing both functions in a single step—i.e., either an immunoprecipitation alone or an immunoblot alone is sufficient to supply the desired information.

The most useful functions for such antibodies are likely to be those that in fact are not possible using traditional reagents. For example, using anti-phosphopeptide antibodies, it is possible to identify and isolate distinct phosphorylated species of a phosphoprotein containing multiple phosphorylation sites. Thus, phosphorylation at each site can be examined independently, and a preparative separation of individual phosphospecies of a holoprotein can be achieved. Another unique application is analysis of the abundance and phosphorylation state of individual proteins in situ in preparations of cells or tissues. Because phosphorylation is a major mode of regulation of protein function, the phosphorylation state is often an indicator of the functional status of a protein. The mere identification of a protein in a cell or tissue specimen gives no indication of its functional status. The ability of anti-phosphopeptide antibodies to demonstrate the phosphorylation state, and by extrapolation the functional state, of a single protein with high specificity places these reagents in a unique class. Hence, identity and functional status may be probed simultaneously using one simple assay. In tissue sections, one may probe to determine whether a particular protein is present, and, if present, in what functional state it is found. The authors have employed this strategy in immunohistochemical staining of formalin-fixed, paraffin-embedded human breast tumors using antibody to the phosphorylated form of the receptor tyrosine kinase Neu (DiGiovanna and Stern, 1995), and were able to demonstrate that Neu is phosphorylated, and therefore functionally active, in only a subset of the tumors that overexpress this protein.

Anti-phosphopeptide antibodies possess unique properties that render them capable of performing functions not possible with conventional reagents. From the technical considerations regarding their production, it would

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follow that antibodies with specificity for the nonphosphorylated state should also be achievable, and the production of such antibodies has also been reported (Czernik et al., 1991; Epstein, 1995; Kawakatsu et al., 1996; Nairn et al., 1982; Roussel, 1990; Tzartos et al., 1995).

Critical Parameters

In the production of polyclonal anti-phosphopeptide antibodies, a major challenge is the depletion of cross-reactivity. The first consideration in achieving this is the anticipation of potential cross-reacting proteins in the planning phase of the procedure. Cross-reactivity is most likely to occur when the target protein is a member of a family of closely related homologous proteins—e.g., of the subfamilies of tyrosine protein kinases. The authors have produced a monoclonal anti-phosphopeptide antibody with specificity for the Tyr-1248 autophosphorylation site of Neu (DiGiovanna and Stern, 1995). A homologous site exists in the epidermal growth factor receptor (EGFR). The antibody produced in the authors' laboratory does not cross-react with the homologous EGFR site in spite of having identical residues in seven of the 14 amino acids of the peptide sequence (five of which are N-terminal to the phosphotyrosine, one C-terminal, and the phosphotyrosine itself). Thus, specificity is achievable even with at least up to 50% identity. Nevertheless, such a close relationship among the phosphorylation sites of different proteins highlights the importance of considering all known related proteins.

The second consideration in depleting cross-reactivity is the quantity of cross-reactive antibodies in a given aliquot to be purified relative to the capacity of the peptide columns used for the negative selection. Because of the expense of the peptides, the authors have generally prepared small columns (with 2- to 3-ml bed volumes) and passed the sera over the columns multiple times, empirically determining the number of passes necessary to quantitatively deplete cross-reacting material. In these columns, 3 µmol of ligand is coupled per milliliter of Affi-Gel 10. The manufacturer states that the resin contains ~15 µmol of active ester per milliliter of gel, and that the gel has a capacity for 35 mg of protein or 15 to 20 µmol of a low-molecular-weight ligand per milliliter of gel. The BSA and phosphotyrosine columns are relatively inexpensive to produce; thus, a single large column of each is practical and sufficient.

Because the authors elute the antibodies from the positive-selection affinity column in strongly chaotropic solutions that are potentially deleterious to the stability of the antibody (3 M NaSCN or 3.5 M followed by 4.5 M $MgCl_2$), care is taken to collect the fractions in prepared dialysis tubing so that they may be immediately placed into the PBS dialysate, thus minimizing the time that the antibodies are exposed to the eluting solutions. Although $MgCl_2$ is thought to be "gentler," the authors have found that gravity-driven flow rates from phosphopeptide columns eluted with this salt quickly become extremely slow, possibly because of precipitation of the salt in the column or an interaction of the Mg^{2+} ion with the phosphate groups. Thus use of NaSCN is preferred, and this salt appears to permit recovery of comparable activity.

Troubleshooting

In the production of polyclonal anti-phosphopeptide antibodies, the most common unfavorable outcomes are persistence of cross-reactivity, loss of specific reactivity, and poor yield. Although it may be tempting, in the interest of saving time, to perform the entire purification and postpone all analyses until the end (a reasonable course of action once a scheme is known to work), it may be prudent, especially with a new protocol, to analyze the serum at each step. For example, the authors have produced antibody to phosphorylated EGFR (M. DiGiovanna, M.A. Lerman, and D. Stern, unpub. observ.). The crude antiserum cross-reacted with the related receptors Neu and HER-4, which had been anticipated. The purification was monitored by probing, after each column, an immunoblot consisting of four lanes containing lysates from cells overexpressing (1) phosphorylated EGFR, (2) nonphosphorylated EGFR, (3) phosphorylated Neu, and (4) phosphorylated HER-4. In following the depletion of cross-reactivity, such stepwise analysis demonstrates whether undesired cross-reactivity has been eliminated or whether further passes through a particular negative-selection column are necessary.

If loss of specific reactivity is also a problem, stepwise analyses can also demonstrate where the loss is occurring. Loss of specific reactivity can occur for three major reasons. The first possible reason is that the antibody is not stable over the course of the purification, which may be the case if the procedure is very prolonged. If this is suspected, the pace of the purification may be hastened or the procedure may be carried out at 4°C instead of room

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temperature. With prolonged purifications, it is especially important to include sodium azide in the PBS used to dilute the serum, to prevent microbial growth. Activity may also be lost nonspecifically as a result of the multiple manipulations in a protocol requiring multiple columns and/or multiple passes through each column. In this case, fraction analysis may indicate which columns are absolutely essential, and also indicate the minimum necessary number of passes through each column. Also, creating larger columns with greater capacity should reduce the number of times the serum will need to be passed over each to deplete cross-reactivity. Finally, specific activity may be lost as a result of the presence in one of the negative-selection columns of a cross-reacting peptide that is so similar to the peptide of interest that essentially any antibody that recognizes the cognate phosphopeptide will recognize this phosphopeptide as well. In this last theoretical scenario, ultimate unique specificity may not be achievable.

The columns used in the purifications can be regenerated and used repeatedly if stored at 4°C in sodium azide–containing buffer to prevent microbial contamination and consequent damage to the matrix. A theoretical consideration is that with repeated use, phosphatases in serum may eventually cleave enough phosphate groups from the peptide moieties on the column to render it ineffective as a phosphopeptide column. Thus, it may be prudent to periodically prepare fresh columns. An alternative would be to add phosphatase inhibitors to the serum prior to purification. Similarly, proteases in serum may eventually cleave peptides from the resin, and this may be prevented by adding protease inhibitors to the serum.

Anticipated Results

The authors have obtained yields of ~1 mg of purified anti-phosphopeptide antibody from a single 15-ml serum sample (M. DiGiovanna, M.A. Lerman, and D. Stern, unpub. observ.). These antibodies have been used in the immunoprecipitation of phosphoproteins from both denaturing and nondenaturing solutions. They have also been used in immunoblotting, in immunofluorescence on fixed cultured cells, and in immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections. Specificity can be demonstrated when such assays are inhibited by cognate phosphopeptide but not by cognate nonphosphopeptide or unrelated phosphopeptides.

Time Considerations

The principal time-consuming step is the production of an immune response in the immunized animals. The authors have obtained high titers of reactive sera as early as 6 to 8 weeks in animals that have been boosted every 2 weeks. Later bleeds with continued boosts have yielded even higher titers. Once adequate bleeds have been obtained and the necessary affinity columns prepared, purification can generally be carried out within 1 week, with the exact length of time depending upon the number of columns and the necessity of analyzing the product after each step.

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Assays of Protein Kinases Using Exogenous Substrates

In studies of the regulation of specific biochemical events by reversible phosphorylation, assaying the protein kinases themselves can often lead to significant progress in understanding the mechanistic details of a system under study. This unit describes assays for a variety of protein kinases that require different conditions to detect and measure their activities—cyclic nucleotide–dependent kinases (see Basic Protocol 1), protein kinase C and isoforms (see Basic Protocol 2), casein kinases (see Basic Protocol 3 and Alternate Protocol), Ca^{2+}/calmodulin–dependent kinases (see Basic Protocol 4), and tyrosine kinase (see Basic Protocol 5). The unit is not meant to be a catalog of individual protein kinase assays; however, the general principles of these assays should apply to most if not all known protein kinases. It is also possible to perform in-gel assays for specific kinase activity (see Basic Protocol 6).

To assay the phosphotransfer reactions catalyzed by protein kinases, it is necessary first to identify a target substrate for the transfer reaction. Essentially this means a substrate that is quite specific—i.e., is not phosphorylated very well or at all by other protein kinases under the appropriate assay conditions (see Strategic Planning). The source of the enzyme activity can be crude cell lysate (see Support Protocol 1), a cellular fraction (see *UNIT 4.3*), an immunoprecipitate, a partially purified protein, or a purified enzyme. The results of the assay can be evaluated using trichloroacetic acid (TCA) precipitation to measure incorporation of radioactivity (see Support Protocol 2), adsorption of the labeled material onto P81 phosphocellulose paper (see Support Protocol 3), or electrophoretic separation and autoradiography (see Support Protocol 4).

STRATEGIC PLANNING

The basic strategy for protein kinase assays involves the use of a labeled donor substrate so that when phosphotransferase activity is present in the enzyme sample, accumulation of the label in the protein or peptide acceptor substrate can be easily detected. The most often used protocol requires [γ-^{32}P]ATP as the donor substrate and a specific protein or peptide as the acceptor substrate. Phosphotransfer is detected as the accumulation of [^{32}P]-labeled protein or peptide. The main problem with this assay is that it requires ^{32}P, a high-energy β emitter. There are a few examples of kinase assays that do not require [γ-^{32}P]ATP, but they are far more specialized assays and generally not as widely applicable. Hopefully, further development of nonradioactive kinase assays will provide good alternatives and reduce the use of radioisotopes.

Enzyme Sources

Of all the choices to be made in designing protein kinase assays, of paramount importance is the source of enzyme activity: very crude sources such as whole-cell lysates (see Support Protocol 1 and *UNIT 13.2*), immunoprecipitates, partially purified preparations, and homogeneous purified protein can be the source of the enzyme activity. The first requirement is that the kinase activity be relatively stable under the conditions used to prepare the enzyme and under the conditions used for the assay. Often enzymes are unstable in whole-cell lysates or homogenates and become very stable only after an initial purification step. Protein kinases commonly exist in intact cells as phosphoproteins themselves, and the phosphorylation state is often important for activity. Thus, it is essential to attempt to preserve the phosphorylation state, e.g., by inhibiting protein phosphatases. Including specific kinase inhibitors in the lysis or homogenation buffer is

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a common procedure to inhibit serine/threonine kinases (sodium fluoride, β-glycerophosphate, okadaic acid) or tyrosine kinases (sodium vanadate, phenylarsinine oxide). Other common inclusions in lysis buffer are chelating agents and protease inhibitors. Many enzymes, including kinases, are sensitive to limited proteolysis. Chelators, such as EDTA and EGTA, chelate calcium and reduce the activity of calcium-activated proteases. The most commonly used protease inhibitors include phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, antipain, and benzamidine. It is always a good idea to add these inhibitors to the lysis buffer just before it is used to lyse cells, especially in the early stages of a new project; the need for specific inhibitors should be investigated at a later time. The oxidation state of cysteines and the state of disulfide linkages may influence enzyme activity, so inclusion of a reducing agent—e.g., 2-mercaptoethanol (2-ME) or dithiothreitol (DTT)—is sometimes essential to preserve enzyme activity. Low concentrations of a detergent such as Brij35, at or just above the critical micelle concentration, may also stabilize enzyme activity.

Choice of Substrate

Selecting a substrate normally depends on the type of experiment and the enzyme preparation, i.e., are there any contaminating kinases in the enzyme sample that phosphorylate the same or similar substrates. The type of experiment may range from following a particular activity through purification to determining substrate specificity or kinetics for the kinase-catalyzed reaction. Most often specific substrate information is available from previously published work or from initial experiments to identify a suitable substrate. It is always helpful to evaluate the suitability of several substrates in parallel experiments in order to identify the best substrate.

For crude extracts, the best substrate will be one that is phosphorylated by the kinase of interest and by no other kinase. In practice this is rarely the case, so a compromise must be reached. If an antibody that recognizes the kinase is available, it is easier to use that antibody to immunoprecipitate the protein and use the immunoprecipitate as the source of enzyme activity. Immunoprecipitation is useful because it provides a quick one-step partial purification of the kinase that also concentrates the activity. With a partially purified enzyme, it may be easier to select an appropriate specific substrate.

Histones are very basic proteins found complexed with DNA in the nucleus of the cell. They have been used for many years as protein kinase substrates because of their relative abundance, ease of purification, and ability to function as very good substrates for a large number of protein kinases. Distinct fractions of mixed histones and purified histones are available commercially from a wide variety of sources. Different kinases have different specificities with respect to phosphorylation of histones, hence the type of histone preparation that should be used depends on the type of kinase to be assayed. Histones have been used to assay many protein kinases and may be used for cAMP- and cGMP-dependent protein kinases. However, phophorylation of histone substrates is generally not enzyme specific—e.g., histone H1 is phosphorylated by cAMP-dependent protein kinases, most cyclin/cdk complexes, and some protein kinase Cs albeit on different sites, and histone 2B is phosphorylated by both cAMP- and cGMP-dependent protein kinases and protein kinase B—so more specific substrates are generally preferred.

Initially, it is much easier to use intact proteins as substrates for kinase assays. However, once a substrate is identified, specific determinant(s) within the protein that allow phosphorylation and specific primary sequence requirements can be identified, and the appropriate peptide sequences modeled on the phosphorylation site can be synthesized and used as substrate.

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Table 13.7.1 Substrate Specificities of Some Protein Serine/Threonine Kinases

Enzyme	Consensus sequence[a,b]
cAMP-dependent protein kinase	XArgArgXSerΦ
cGMP-dependent protein kinase	XArg/LysXSerΦ
Protein kinase Cα	XLys/ArgXXSerX
Cyclin B/cdc2	XSerProXLys/ArgX
Casein kinase I	Glu/Ser(P) XXSer
Ca^{2+}/calmodulin–dependent protein kinase type II	XArgXXSerX
MAP kinase	XProSerXX
Casein kinase II	XSerXGluGlu/AspX

[a]See Table A.1A.1 for amino acid codes. X indicates any amino acid, Φ indicates a hydrophobic residue, underlining indicates the sites of phosphorylation (which, although marked Ser in each case, may be either Ser or Thr residues), and (P) indicates a phosphorylated residue.

[b]Sequence information based on Pearson and Kemp (1991).

Table 13.7.2 Substrate Specificities of Some Protein Tyrosine Kinases

Enzyme	Optimal sequence[a,b]
MT/c-src	GluGluIleTyrGlyGluPheGlu
c-abl	AlaGluValIleTyrAlaAlaProPhe
c-fps/fes	GluGluGluIleTyrGluGluIleGlu
lck	XGluXIleTyrGlyValLeuPhe
EGF receptor	GluGluGluGluTyrPheGluLeuVal
PDGF receptor	GluGluGluGluTyrValPheIleGlu
Insulin receptor	XGluGluGluTyrMetMetMetMet

[a]These sequences were determined through the use of peptide libraries (Songyang et al., 1995), and their relevance to in vivo phosphorylation specificities is unclear.

[b]See Table A.1A.1 for amino acid codes. X indicates any amino acid; underlining indicates the site of phosphorylation.

When synthetic peptide substrates are used to assay protein kinase activity, it is possible to study the specificity of different kinases. Synthetic peptide substrates are available from a number of suppliers, e.g., Peninsula Labs, Bachem Biosciences, and Calbiochem-Novabiochem. Peptides are easy to use: they are potentially available in large quantities as chemically defined molecules, their purification is usually straightforward, and they provide a single phosphorylation site. In addition, it is relatively simple to synthesize a series of peptides with single amino acid changes to further define the specificity of an enzyme. However, peptides do have their disadvantages. They are expensive, $10 to $20 per residue. The kinase may not recognize the disordered structure usually adopted by small peptides in solution, or it may promiscuously phosphorylate the peptide. Finally, it is very difficult to use peptides to model reactions in which prior derivitization (i.e., phosphorylation) is required for specific kinase activity.

Table 13.7.1 summarizes the sequence specificity of a number of protein serine/threonine kinases, and Table 13.7.2 provides similar information for protein tyrosine kinases.

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Assay Conditions

The assay conditions can have profound effects on any enzyme activity: if pepsin is assayed at pH 7, it is essentially inactive, but at pH 3, it has good protease activity. Similarly T4 DNA ligase is inhibited by salt concentrations >150 mM. For kinases, it is a good idea to use preliminary experiments to identify the effects of pH, salt concentration, concentrations of cations such as Mg^{2+} or Mn^{2+}, and temperature to optimize the assay conditions for the kinase of interest. It is often advantageous to perform kinase assays at 30°C rather than 37°C because the lower temperature makes it easier to stay within the linear range of the kinase, thus providing more control of the assay.

Another factor to be considered when optimizing an assay for a newly identified kinase is the concentration of ATP in the reaction mixture. In these protocols and in general, detection of kinase activity is based on the transfer of radiolabeled phosphate from ATP to the substrate, so the concentration of ATP can be varied only a little. Also, the specific activity of the [γ-^{32}P]ATP must be known in order to actually measure phosphotransfer. Most kinases have a K_M for ATP of 1 to 100 μM. If there is too much ATP in the reaction mixture, it will be difficult to measure phosphotransfer. An ATP concentration of 50 to 100 μM tends to work well. At that concentration, the enzyme should be working at ≥50% of maximum, depending on its apparent K_M for ATP, and addition of sufficient [γ-^{32}P]ATP to measure phosphotransfer is not prohibitively expensive. Usually the substrate concentration is high so that the enzyme is working at or close to V_{max}.

Controls

Including the correct controls for a kinase assay is critical to the success of the assay, especially when the enzyme source is a cell or tissue extract. The appropriate controls should always include a no substrate control, a no enzyme source control, a heat-denatured enzyme control, and, for enzymes that require an activator or cofactor, controls that use irrelevant activator or cofactor and controls without activator or cofactor.

CAUTION: These assays use [γ-^{32}P]ATP which should be handled and disposed of according to safety regulations. See *APPENDIX 2B* and the institutional Radiation Safety Office for guidelines for proper handling and disposal of ^{32}P.

BASIC PROTOCOL 1

ASSAY FOR CYCLIC NUCLEOTIDE–DEPENDENT PROTEIN KINASES

Cyclic adenosine monophosphate (cAMP)–and cyclic guanosine monophosphate (cGMP)–dependent protein kinases (PrKs) are quite similar in structure and require similar assay conditions. cAMP-PrK is a heterotetramer consisting of two regulatory (R) subunits and two catalytic (C) subunits, R_2C_2. In each case the regulatory subunits have binding sites for two molecules of cAMP. When the cAMP concentration is elevated in cells after activation of receptor-linked adenylyl cyclases, cAMP binds to the R subunits, causing the affinity of the C subunits for R subunits to drop by about four orders of magnitude. The formation of R_2cAMP_4 dimers and free C-subunits is favored, and the C-subunits are now active.

The case of cGMP-PrK is slightly different in that there are no free catalytic subunits released. cGMP-PrK is composed of two identical subunits. Each subunit has a regulatory domain and a catalytic domain, which are homologous to the R and C subunits of cAMP-PrK. On binding four molecules of cGMP the enzyme is activated, presumably by a conformational change.

The assay procedures for these two kinases are very similar and the same protein substrate can be used for each one as detailed below. More recently, both of these kinases have been assayed using peptide substrates.

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Materials

5× cyclic nucleotide–dependent kinase reaction buffer (see recipe)
10 mg/ml histone 2B in H_2O
[γ-^{32}P]ATP solution (see recipe)
20× cyclic nucleotide solution: 20 µM cyclic AMP in H_2O/20 µM cyclic GMP in H_2O
Enzyme sample containing cyclic nucleotide–dependent kinase activity (see Support Protocol 1), kept on ice until use
30° water bath

Additional reagents and equipment for TCA precipitation (see Support Protocol 2), adsorption onto P81 phosphocellulose paper (see Support Protocol 3), or electrophoretic analysis (see Support Protocol 4)

1. For each assay reaction, add the following to a 1.5-ml microcentrifuge tube kept on ice:

   4 µl 5× cyclic nucleotide–dependent kinase reaction buffer
   1 µl of 10 mg/ml histone 2B
   1 µl [γ-^{32}P]ATP solution (to give 5 µCi/µl and 5 µM ATP final)
   1 µl 20× cyclic nucleotide solution
   0 to 13 µl H_2O.

   Cap tube and warm the mixture in a 30°C water bath.

   Perform each assay in triplicate and include no substrate, no enzyme, and no cyclic nucleotide controls.

   The total reaction mix volume is 20 µl per reaction. The amount of water required depends on how much enzyme sample is used.

   Sufficient reaction mix can be prepared in a single tube for all the reactions by multiplying the quantities for a single reaction by the total number of reactions + 1. Then the total amount of reaction mixture (minus enzyme) for one reaction can be added to each tube. This is often very convenient and reduces the potential for pipetting errors.

2. Start the reaction by adding 1 to 14 µl ice-cold enzyme sample containing cyclic nucleotide–dependent kinase activity.

   The volume of enzyme used depends on the amount of activity in the enzyme sample. In a preliminary experiment, the maximum indicated amount can be used to gauge the extent of the phosphotransfer reaction. The volume can be reduced as appropriate in order to allow for linear incorporation of phosphate into the substrate during the assay.

   For enzyme samples that are immunoprecipitates adsorbed onto Sepharose beads, prepare the reaction mix minus enzyme source first, warm it, then dispense it into the immunoprecipitate. For immunoprecipitates, it is advisable to scale up the reaction for a total volume of 100 µl: add 75 µl reaction mix to 25 µl immunoprecipitate bound to beads.

3. Incubate 10 min in a 30°C water bath.

4. Stop the reaction using the reagent appropriate for the analytic method to be used—20 µl ice-cold 10% TCA for TCA precipitation (see Support Protocol 2), or 10 µl or 20 µl ice-cold 2× SDS-PAGE sample buffer for electrophoretic analysis (see Support Protocol 4). Use 10 µl of the reaction mix for adsorption to P81 phosphocellulose paper (see Support Protocol 3). Proceed with analysis by one of those methods.

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ASSAY FOR PROTEIN KINASE C ISOFORMS

There are presently at least ten members of the protein kinase C (PKC) family of kinases. Protein kinase C was originally identified as a kinase activity that was reversibly activated by calcium (Ca^{2+}), phospholipid (usually phosphatidylserine), and the neutral lipid diacylglycerol (DAG; Takai et al., 1979). Subsequently, these kinases were shown to be the sites of action for the tumor-promoting phorbol esters, which bind to some PKC isoforms and activate the kinase activity by substituting for DAG.

The initial isoforms of PKC that were purified were called α, β, and γ, and it is these forms that are the most clearly regulated by Ca^{2+}, phospholipid, and DAG or phorbol esters. They are termed the "conventional" subfamily of PKCs (cPKCs). Another subfamily contains δ, ε, ν, and θ, the "novel" PKCs (nPKCs); these kinases are not regulated by Ca^{2+} but are activated by phorbol esters and phospholipids. A third subfamily, the "atypical" PKCs (aPKCs), consist of ζ, λ, and ι; these are not regulated by Ca^{2+} nor do they bind phorbol esters or DAG, but there is still a requirement for phospholipids. When the isoforms are analyzed, the reaction buffer must include the appropriate activating cofactors.

Materials

5× PKC reaction buffer (see recipe)
10 mg/ml histone H1 in H_2O
[γ-^{32}P]ATP solution (see recipe)
Enzyme sample containing PKC activity (see Support Protocol 1)
30°C water bath

Additional reagents and equipment for TCA precipitation (see Support Protocol 2), adsorption onto P81 phosphocellulose paper (see Support Protocol 3), or electrophoretic analysis (see Support Protocol 4)

1. For each assay reaction, add the following to a 1.5-ml microcentrifuge tube:

   4 µl 5× PKC reaction buffer
   1 µl 10 mg/ml histone H1
   1 µl [γ-^{32}P]ATP solution (to give 5 µCi/µl and 5 µM ATP final)
   0 to 14 µl H_2O.

   Cap tube and warm the mixture 10 min in a 30°C water bath.

   Perform each assay in triplicate and include no substrate and no enzyme controls.

   The total reaction mix volume is 20 µl per reaction. The amount of water required depends on how much enzyme sample is used.

   Sufficient reaction mix can be prepared in a single tube for all the reactions by multiplying the quantities for a single reaction by the total number of reactions + 1. Then the total amount of reaction mixture (minus enzyme) for one reaction can be added to each tube. This is often very convenient and reduces the potential for pipetting errors.

2. Start reaction by adding 1 to 14 µl enzyme sample containing PKC activity to the warmed reaction mixture.

   The volume of enzyme used depends on the amount of activity in the enzyme sample. In a preliminary experiment, the maximum indicated amount can be used to gauge the extent of the phosphotransfer reaction. The volume can be reduced as appropriate in order to allow for linear incorporation of phosphate into the substrate during the assay.

   For enzyme samples that are immunoprecipitates adsorbed onto Sepharose beads, prepare the reaction mix minus enzyme source first, warm it, then dispense it into the immunoprecipitate. For immunoprecipitates, it is advisable to scale up the reaction for a total volume of 100 µl: add 75 µl reaction mix to 25 µl immunoprecipitate bound to beads.

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3. Incubate 10 min in a 30°C water bath.

4. Stop the reaction using the reagent appropriate for the analytic method to be used—20 µl ice-cold 10% TCA for TCA precipitation (see Support Protocol 2), or 10 µl, or 20 µl ice-cold 2× SDS-PAGE sample buffer for electrophoretic analysis (see Support Protocol 4). Use 10 µl of the reaction mix for adsorption to P81 phosphocellulose paper (see Support Protocol 3). Proceed with analysis by one of those methods.

ASSAY FOR CASEIN KINASES USING β-CASEIN

BASIC PROTOCOL 3

Casein is a very acidic milk protein available in reasonable purity and quantity. Casein kinases were named for their ability to phosphorylate casein rather than other substrates available at the time. Because of its acidic nature, the use of casein as a substrate presents a number of problems, one of which is the fact that it does not carry a net positive charge at low pH and thus does not bind to P81 phosphocellulose paper. In recent years this problem has been circumvented by using short acidic peptides engineered to bind to P81 paper as substrates for the casein kinases (see Alternate Protocol).

There are currently two known mammalian casein kinases, casein kinase I and casein kinase II, named for their order of elution from anion-exchange chromatography columns. Casein kinases show a marked preference for substrates containing acidic residues close to the serine or threonine to be phosphorylated.

Casein kinase I is a 37-kDa monomer that phosphorylates serine and threonione residues (see Table 13.7.1) and utilizes ATP only as phosphoryl group donor. The basic phosphorylation consensus sequence is Glu/Ser(P)XXSer (where the underlined residue is the phosphate acceptor, and may be either Ser or Thr).

Mammalian casein kinase II is a tetramer of $\alpha_2\beta_2$ structure: the α-subunit is 24 to 28 kDa and the β-subunit is 37 to 44 kDa. Its preferred substrate has aspartate or glutamate residues C-terminal to the phosphorylation site serine or threonine and a consensus of SerGluGlu/Asp. Casein kinase II is unusual in that it can utilize both ATP and GTP as phosphoryl group donor with similar K_M values, ~10 µM for ATP and ~30 µM for GTP. Mammalian casein kinase II is also inhibited by heparin, whereas casein kinase I is not. The IC_{50} for the inhibition of casein kinase II by heparin is in the 10 to 20 nM range and at these concentrations casein kinase I is not inhibited.

Assays of either casein kinase I or II can be performed using casein as substrate. When isolated from milk, casein is a phosphoprotein. However, it is preferable to use dephosphorylated casein as substrate; this is commercially available in a relatively pure state as either the α or β isoform.

Materials

5× casein kinase reaction buffer (see recipe)
10 mg/ml β-casein in H_2O
[γ-^{32}P]ATP solution (see recipe)
Enzyme sample containing casein kinase activity (see Support Protocol 1), kept on ice
30°C water bath
Additional reagents and equipment for TCA precipitation (see Support Protocol 2) or electrophoretic analysis (see Support Protocol 4)

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1. For each assay reaction, add the following to a 1.5-ml microcentrifuge tube kept on ice:

   4 µl 5× casein kinase reaction buffer
   1 µl 10 mg/ml β-casein
   1 µl [γ-^{32}P]ATP solution (to give 5 µCi/µl and 5 µM ATP final)
   1 to 14 µl H_2O.

   Cap tube and warm the mixture 10 min in a 30°C water bath.

   This reaction mix can also be used for α-casein.

   The total reaction mix volume is 20 µl per reaction. The amount of water required depends on how much enzyme sample is used.

   Sufficient reaction mix can be prepared in a single tube for all the reactions by multiplying the quantities for a single reaction by the total number of reactions + 1. Then the total amount of reaction mixture (minus enzyme) for one reaction can be added to each tube. This is often very convenient and reduces the potential for pipetting errors.

2. Start the reaction by adding 1 to 14 µl enzyme sample containing casein kinase activity.

   The volume of enzyme used depends on the amount of activity in the enzyme sample. In a preliminary experiment, the maximum indicated amount can be used to gauge the extent of the phosphotransfer reaction. The volume can be reduced as appropriate in order to allow for linear incorporation of phosphate into the substrate during the assay.

   For enzyme samples that are immunoprecipitates adsorbed onto Sepharose beads, prepare the reaction mix minus enzyme source first, warm it, then dispense it into the immunoprecipitate. For immunoprecipitates, it is advisable to scale up the reaction for a total volume of 100 µl: add 75 µl reaction mix to 25 µl immunoprecipitate bound to beads.

3. Incubate 10 min in a 30°C water bath.

4. Stop the reaction using the reagent appropriate for the analytic method to be used—20 µl ice-cold 10% TCA for TCA precipitation (see Support Protocol 2), or 10 µl or 20 µl ice-cold 2× SDS-PAGE sample buffer for electrophoretic analysis (see Support Protocol 4). Proceed with analysis by one of those methods.

ALTERNATE PROTOCOL

ASSAY FOR CASEIN KINASES USING A PEPTIDE SUBSTRATE

Casein kinase I can be assayed with the peptide AspAspAspGluGluSerIleThrArgArg. Casein kinase II has most recently been assayed using specific peptide substrates, e.g., ArgArgArgGluGluGluThrGluGluGlu (where the underlined residue is the phosphate acceptor). In both cases the arginine residues allow binding to P81 phosphocellulose paper. The first peptide is relatively specific for casein kinase I, but the kinetics of its phosphorylation are not ideal; the K_M is in the millimolar range. Thus, it is preferable to use this assay for casein kinase I only if the kinase is highly purified.

Additional Materials (also see Basic Protocol 3)

10 mM synthetic peptide substrate solution (see recipe) for casein kinase: e.g., AspAspAspGluGluSerIleThrArgArg (for casein kinase I) or ArgArgArgGluGluGluThrGluGluGlu (for casein kinase II)

Additional reagents and equipment for TCA precipitation (see Support Protocol 2), adsorption onto P81 phosphocellulose paper (see Support Protocol 3), or electrophoretic analysis (see Support Protocol 4)

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1. For each assay, add the following to a microcentrifuge tube:

   10 µl 5× casein kinase reaction buffer
   5 µl 10 mM synthetic peptide substrate solution for casein kinase
   5 µl [γ-^{32}P]ATP solution (to give 5 µCi/µl and 5 µM ATP final)
   0 to 30 µl H_2O.

   Cap the tube and warm 10 min in a 30°C water bath.

   The total reaction mix volume is 50 µl per reaction. The amount of water required depends on how much enzyme sample is based.

   Sufficient reaction mix can be prepared in a single tube for all the reactions by multiplying the quantities for a single reaction by the total number of reactions + 1. Then the total amount of reaction mix minus enzyme for one reaction can be added to each tube. This is often very convenient and reduces the potential for pipetting errors.

2. Start the reaction by adding 1 to 30 µl enzyme sample containing casein kinase activity.

   The volume of enzyme used depends on the amount of activity in the enzyme sample. In a preliminary experiment, the maximum indicated amount can be used to gauge the extent of the phosphotransfer reaction. The volume can be reduced as appropriate in order to allow for linear incorporation of phosphate into the substrate during the assay.

   For enzyme samples that are immunoprecipitates adsorbed onto Sepharose beads, prepare the reaction mix minus enzyme source first, warm it, then dispense it into the immunoprecipitate. For immunoprecipitates, it is advisable to scale up the reaction for a total volume of 100 µl: add 75 µl reaction mix to 25 µl immunoprecipitate bound to beads.

3. Incubate 10 min in a 30°C water bath.

4. Stop the reaction using the reagent appropriate for the analytic method to be used—20 µl ice-cold 10% TCA for TCA precipitation (see Support Protocol 2), or 10 µl or 20 µl ice-cold 2× SDS-PAGE sample buffer for electrophoretic analysis (see Support Protocol 4). Use 10 µl of the reaction mix for adsorption to P81 phosphocellulose paper (see Support Protocol 3). Proceed with analysis by one of those methods.

ASSAY FOR Ca^{2+}/CALMODULIN–DEPENDENT KINASES

BASIC PROTOCOL 4

As the name implies, Ca^{2+}/calmodulin–dependent kinases are dependent on Ca^{2+} and calmodulin for their activity. Ca^{2+}/calmodulin–dependent kinase I (CaM kinase I) is a monomeric protein of ~40 kDa. Ca^{2+}/calmodulin–dependent kinase II (CaM kinase II) is composed of the products of four distinct genes for α, β, γ, and δ subunits. In its native form, CaM kinase II is an oligomer of M_r ~500,000 to 600,000. As its name suggests, both Ca^{2+} and calmodulin are required for activity.

Little is known of the physiological function of CaM kinase I. Activity can be assayed using the protein synapsin 1 as substrate, but both CaM kinase 1 and CaM kinase II phosphorylate full-length synapsin 1. This problem can be overcome by using synthetic peptide substrates that include the specific phosphorylation site for the enzyme being assayed. CaM kinase I can be assayed using a peptide containing site 1 of synapsin I, TyrLeuArgArgArgLeuSerAspSerAsnPhe (where the underlined residue is the phosphate acceptor). CaM kinase II can be assayed using autocamtide, LysLysAlaLeuArgGlnGluThrValAspAlaLeu (Hanson et al., 1989), which is modeled on the autophosphorylation site of the kinase itself.

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Materials

5× CaM kinase reaction buffer (see recipe)
10 mM synthetic peptide substrate solution (see recipe): e.g., TyrLeuArgArgArgLeuSerAspSerAsnPhe (for CaM kinase I) or LysLysAlaLeuArgGlnGluThrValAspAlaLeu (autocamtide for CaM kinase II)
[γ-^{32}P]ATP solution (see recipe)
1 mg/ml calmodulin in Milli-Q-purified water (store small aliquots at –70°C)
Enzyme sample containing CaM kinase activity (see Support Protocol 1), kept on ice
30°C water bath

Additional reagents and equipment for adsorption onto P81 phosphocellulose paper (see Support Protocol 3)

1. Set up and label duplicate 1.5-ml microcentrifuge tubes. For each reaction, prepare a mixture containing:

   5 µl 5× CaM kinase reaction buffer
   5 µl 10 mM synthetic peptide substrate solution
   5 µl [γ-^{32}P]ATP solution (to give 5 µCi/µl and 5 µM ATP final)
   5 µl 1 mg/ml calmodulin *or* 5 µl H_2O
   0 to 30 µl H_2O.

   Cap tube and warm the mixture 10 min in a 30°C water bath.

   The reaction mix is described for 50 µl per reaction, but it can be carried out in volumes of 25 to 100 µl by adjusting the amounts of the ingredients proportionally.

   Control reactions should include one reaction without calmodulin so that any background phosphotransfer reactions can be accounted for and only calcium^{2+}/calmodulin–dependent phosphotransfer is measured.

2. Start each set of reactions sequentially every 30 sec by adding 1 to 25 µl enzyme sample containing CaM kinase activity to one of the tubes.

   The volume of enzyme used depends on the amount of activity in the enzyme sample. In a preliminary experiment, the maximum indicated amount can be used to gauge the extent of the phosphotransfer reaction. The volume can be reduced as appropriate in order to allow for linear incorporation of phosphate into the substrate during the assay.

   For enzyme samples that are immunoprecipitates adsorbed onto Sepharose beads, prepare the reaction mix minus enzyme source first, warm it, then dispense it into the immunoprecipitate. For immunoprecipitates, it is advisable to scale up the reaction for a total volume of 100 µl: add 75 µl reaction mixture to 25 µl immunoprecipitate bound to beads.

3. Incubate for 10 min in a 30°C water bath.

4. Once the desired incubation time has elapsed, pipet 30 µl of the contents of the tube quickly onto the surface of prepared P81 paper squares (see Support Protocol 3).

   Only adsorption onto P81 phosphocellulose (see Support Protocol 3) is used to measure CaM kinase–mediated transfer because peptides are not efficiently precipitated by TCA, and they require specialized techniques for analysis by PAGE.

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ASSAY FOR TYROSINE KINASES

Acid-denatured rabbit muscle enolase was first used as an exogenous tyrosine kinase substrate in the early 1980s, and it is still a useful substrate in a number of experimental situations. Usually enolase is used as a substrate for cytosolic tyrosine kinases such as $pp60^{c\text{-}src}$. Once the reaction is complete, phosphorylation of enolase is detected by separating proteins in the reaction mixture by SDS-PAGE (*UNIT 10.1*).

The following method is optimized for $pp60^{c\text{-}src}$, but may be applied to other tyrosine kinases. It is also useful as a framework for the assay of any tyrosine kinase, but optimization of the immunoprecipitation and assay buffers is required for the tyrosine kinase of interest.

Materials

Rabbit muscle enolase (enolase EC 4.2.1.11; ammonium sulfate precipitate or suspension purified from rabbit muscle)
1 mM DTT/50 mM HEPES, pH 7.0
Glycerol
100 mM acetic acid
5× tyrosine kinase reaction buffer (see recipe)
[γ-^{32}P]ATP solution (see recipe)
Enzyme sample containing tyrosine kinase activity (see Support Protocol 1), kept on ice
2× SDS-PAGE sample buffer (*UNIT 10.1*), ice-cold
30°C and boiling water bath

Additional reagents and equipment for SDS-PAGE (see Support Protocol 4 and *UNIT 10.1*)

1. Microcentrifuge the equivalent of 100 µg rabbit muscle enolase 5 min at maximum speed, 4°C. Discard the supernatant.
2. Add 10 µl of 1 mM DTT/50 mM HEPES, pH 7.0, to the pellet, mix thoroughly, and incubate 30 to 60 min on ice.
3. Add 10 µl glycerol, mix, and then keep on ice if assays are to be performed the same day.

   This preparation can also be stored at −70°C until required.

4. Immediately prior to assay, add 20 µl of 100 mM acetic acid to the enolase solution and mix thoroughly. Incubate this mixture 5 min in a 30°C water bath, and then keep on ice until the reaction mixture is prepared.

   Do not store acid-denatured enolase on ice in this state for >1 hr.

5. For each reaction, add the following to a 1.5-ml microcentrifuge tube:

   4 µl 5× tyrosine kinase reaction buffer
   1 µl acid-denatured enolase (step 4; 2.5 µg enolase total)
   1 µl [γ-^{32}P]ATP (to give 5 µCi/µl and 5 µM ATP final)
   0 to 14 µl H_2O.

   Cap the tube and warm the mixture 10 min in a 30°C water bath.

   The amount of water required depends on the amount of enzyme sample to be added; the total reaction volume should be 20 µl.

   Sufficient reaction mix can be prepared in a single tube for all the reactions by multiplying the quantities for a single reaction by the total number of reactions + 1. Then the total

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amount of reaction mix minus enzyme for one reaction can be added to each tube. This is often very convenient and reduces the potential for pipetting errors.

6. Start the reactions by adding 1 to 14 µl enzyme sample containing tyrosine kinase activity.

   The volume of enzyme used depends on the amount of activity in the enzyme sample. In a preliminary experiment, the maximum indicated amount can be used to gauge the extent of the phosphotransfer reaction. The volume can be reduced as appropriate in order to allow for linear incorporation of phosphate into the substrate during the assay.

   For enzyme samples that are immunoprecipitates adsorbed onto Sepharose beads, prepare the reaction mix minus enzyme source first, warm it, then dispense it into the immunoprecipitate. For immunoprecipitates, it is advisable to scale up the reaction for a total volume of 100 µl: add 75 µl reaction mixture to 25 µl immunoprecipitate bound to beads.

7. Incubate the tube 10 min in a 30°C water bath.

8. Stop the reaction by adding 20 µl ice-cold 2× SDS-PAGE sample buffer. Mix thoroughly and heat the tube 3 min in a boiling water bath. Analyze 20 µl of the reaction by SDS-PAGE (see Support Protocol 4 and *UNIT 10.1*).

   Most tyrosine kinases autophosphorylate on a number of tyrosine residues and in in vitro assays are promiscuous with respect to substrate. Backgrounds are often very high so TCA precipitation and adsorption to P81 phosphocellular paper are not very useful for analysis of results. SDS-PAGE (see Support Protocol 4) gives more specific and cleaner results.

BASIC PROTOCOL 6

IN-GEL PROTEIN KINASE ASSAYS

In-gel protein kinase assays (Kameshita and Fujisawa, 1989) involve the copolymerization of a kinase substrate in the separating gel layer of an SDS-PAGE gel. Protein samples are run on the modified gel as usual (*UNIT 10.1*). After electrophoresis is complete, the separated proteins are allowed to refold by various gel treatments. The gel is then incubated with [γ-^{32}P]ATP at 30°C for enough time to allow the ATP to penetrate the entire gel. After further washes to remove unincorporated [γ-^{32}P]ATP, the gel is stained and fixed as normal. ^{32}P-containing proteins are revealed by autoradiography. Although in general these assays are not particularly sensitive, they are sometimes very useful for identifying kinases in complex mixtures.

Materials

10 mg/ml kinase substrate: e.g., myelin basic protein
Enzyme sample containing kinase activity (see Support Protocol 1), kept on ice
20% (v/v) 2-propanol/50 mM Tris·Cl (pH 8.0 at room temperature; *APPENDIX 2E*)
1 mM DTT/50 mM Tris·Cl (pH 8.0 at room temperature)
6 M guanidine·HCl/1 mM DTT/50 mM Tris·Cl (pH 8.0 at room temperature) *or* 8 M urea/1 mM DTT/50 mM Tris·Cl (pH 8.0 at room temperature)
1 mM DTT/0.05% (v/v) Tween 20/50 mM Tris·Cl (pH 8.0 at 4°C)
Appropriate kinase reaction buffer (see recipes)
10 mM Mg/ATP solution (see recipe)
10 mCi/ml [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, DuPont NEN, or ICN Biomedicals)
5% (w/v) trichloroacetic acid (TCA)
1% (w/v) sodium pyrophosphate/5% (w/v) TCA

Container for radioactive incubation: e.g., small tray with tight cover, or heat-sealable polyethylene bag (Seal-a-Meal)
Seal-a-Meal apparatus (optional)

Additional reagents and equipment for SDS-PAGE (*UNIT 10.1*)

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CAUTION: Because the samples are radioactive, great care should be exercised in sample preparation and loading. Perform the reactions and subsequent manipulations in screw-cap microcentrifuge tubes to minimize the risk of ^{32}P contamination. Run gels until the dye front passes out of the gel, as all residual [γ-^{32}P]ATP will migrate with the dye front. This means that the SDS-PAGE running buffer will be contaminated with ^{32}P, and it should be disposed of as radioactive waste according to safety regulations. See *APPENDIX 2B* for proper handling and disposal.

Separate proteins on modified gel

1. Prepare the mixture for a polyacrylamide gel of the appropriate %T (see *UNIT 10.1*). Add 10 mg/ml kinase substrate to give a final concentration of 1 mg/ml in the gel mixture. Polymerize as usual in a cassette prepared for casting the gel.

   Myelin basic protein has been used with some success for assays of the MAP kinase family of enzymes. However, any protein is suitable if it is a substrate for the kinase of interest. This assay can also be used to identify potential substrates.

2. Prepare the enzyme samples containing kinase activity for SDS-PAGE in the usual way and carry out electrophoresis (see *UNIT 10.1*).

3. Once separation is complete, place the gel in 200 ml of 20% 2-propanol/50 mM Tris·Cl, pH 8.0. Wash the gel for 20 min, change to fresh buffer, and repeat twice for a total of three 20-min washes.

   Washing with propanol removes SDS from the gel.

4. Wash the gel with 200 ml of 1 mM DTT/50 mM Tris·Cl (pH 8.0) three times, 20 min each.

5. Wash the gel with 250 ml of either 6 M guanidine·HCl/1 mM DTT/50 mM Tris·Cl (pH 8.0), *or* 8 M urea/1 mM DTT/50 mM Tris·Cl (pH 8.0) twice, 30 min each.

   Either guanidine·HCl or urea can be used to denature the proteins. Depending on the kinase, one or the other may be more successful; guanidine·HCl should be tried first, because it seems to work best for most kinases.

6. Wash the gel eight to ten times at 4°C over a period of 18 hr with 250 ml of 1 mM DTT/0.05% Tween 20/50 mM Tris·Cl (pH 8.0), to renature proteins.

Assay for kinase activity

7. Incubate the gel with 250 ml of an appropriate kinase reaction buffer containing 10 mM $MgCl_2$, for 20 min at 30°C.

8. Remove all traces of buffer and place the gel in a small tray or a Seal-a-Meal bag (the container is to be used for radioactive incubation). Add as small an amount of kinase reaction buffer as possible to just cover the gel. Add 1/4 of the reaction buffer volume of 10 mM Mg/ATP solution to give a final concentration of 50 µM ATP. Add 20 µCi/ml [γ-^{32}P]ATP.

9. Incubate 1 hr at 30°C.

   The duration of this incubation can be adjusted after viewing the results of the initial experiment.

10. Incubate the gel in 250 ml 5% TCA twice, 15 min each. Wash 10 min in 500 ml of 1% sodium pyrophosphate/5% TCA. Repeat this wash until the solution contains little or no radioactivity.

11. Dry the gel onto filter paper and autoradiograph.

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SUPPORT PROTOCOL 1

PREPARING A CELL LYSATE FOR KINASE ASSAYS

This protocol describes a method for detergent-induced cell lysis to prepare a crude extract containing kinase enzyme activity. Other methods of cell lysis may be appropriate. Hypotonic lysis and isolation of P100 and S100 fractions can also provide useful data on the recovery of a protein kinase as a soluble or membrane-bound activity.

Materials

Cultured cells: adherent cells at ~70% confluence in 100-mm tissue culture dishes or suspension cells at 10^6 cells/ml
PBS (*APPENDIX 2D*), ice-cold
Lysis buffer (see recipe)
Protease inhibitor stock solutions (see recipe)
Microcentrifuge, 4°C

1. Wash the cultured cells twice in ice-cold PBS. Completely aspirate the final wash solution.
2. Add 0.75 ml lysis buffer to ~2×10^7 cells; for adherent cells check that the monolayer is completely covered. Add protease inhibitors from stock solutions to the appropriate final concentration: 1 mM PMSF, 100 µM benzamidine, 5 µg/ml leupeptin, 5 µg/ml pepstatin A, and 5 µg/ml antipain.

   A 0.75-µl lysate should provide sufficient material for immunoprecipitation (use 25% to 50% of the lysate for an initial experiment and scale up or down depending on the results) or simple purification. If the purification procedure involves more than one chromatography step, prepare a larger lysate because only 10% to 20% of the activity is recovered from each chromatography step.

3. Incubate the cells 10 min at 4°C. Scrape the cells from the dish and transfer the lysate to a labeled microcentrifuge tube.
4. Microcentrifuge the lysate 10 min at maximum speed, 4°C. Carefully remove the supernatant and transfer it to a clean microcentrifuge tube.

   Lysates should be used immediately until proper storage conditions (e.g., –70°C, liquid nitrogen) can determined experimentally. For some kinases, it may not be possible to store the lysate at all.

SUPPORT PROTOCOL 2

TCA PRECIPITATION TO DETERMINE INCORPORATION OF RADIOACTIVITY

One of the classical methods for separating a reaction product from the reactants is differential precipitation. In the case of protein kinase assays using a protein substrate and [γ-^{32}P]ATP, it is very easy to precipitate the protein and leave the [γ-^{32}P]ATP in the soluble fraction. Most proteins are quantitatively precipitated by trichloroacetic acid (TCA), so TCA can be used to precipitate proteins phosphorylated during a kinase assay. Protein precipitate can be captured by filtration and the filter can be washed with TCA. TCA precipitation is a quick and reproducible way to determine the extent of [^{32}P]phosphate transfer to protein substrates. However, peptides are not precipitated by TCA, so adsorption of labeled peptides to P81 phosphocellulose paper (see Support Protocol 3) is a more suitable method for analyzing the results of kinase assays that use a peptide as substrate.

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Materials

Assay samples (see Basic Protocols 1 to 5 and Alternate Protocol)
5% and 10% (w/v) trichloroacetic acid (TCA), ice-cold
95% ethanol
Diethyl ether

Whatman GF-C glass-fiber filters
Vacuum manifold (e.g., Fisher)
20-ml scintillation vials
Scintillation counter

1. Stop the reaction by adding 20 µl ice-cold 10% TCA. Mix thoroughly and incubate 10 min on ice to precipitate protein.
2. Pipet sample onto Whatman GF-C glass-fiber filter held in a vacuum manifold. Allow the sample to pass through the filter. Rinse the tube with 500 µl ice-cold 5% TCA. Add this wash to the appropriate filter.
3. Wash each filter with 5 ml ice-cold 5% TCA solution four times. Wash once with 10 ml of 95% ethanol, then three times with 10 ml diethyl ether. Allow each filter to dry for a few minutes.

   CAUTION: *Diethyl ether is extremely flammable and washes should be performed in a fume hood away from any flame or heat source. Ether washes should be disposed of in an appropriate manner.*

4. Place the dry filters in a 20-ml scintillation vial and count in a scintillation counter by detection of Cerenkov radiation.

   Alternatively, if dpm quantitation is desired, place dry filter in 10 ml scintillation fluid and count.

   The filter can also be counted by Cerenkov radiation after the washes with 95% ethanol. If the filters are to be counted in scintillant, however, the diethyl ether washes are essential, because TCA (which is removed by the ether) causes significant chemiluminescence when placed in scintillation fluid, making the counts produced by the scintillation counter meaningless.

ADSORPTION ONTO P81 PHOSPHOCELLULOSE PAPER

SUPPORT PROTOCOL 3

One of the main methods to separate [^{32}P]-labeled proteins or peptides from [γ-^{32}P]ATP after a protein kinase assay reaction is by adsorbing the protein or peptide to P81 phosphocellulose paper. P81 paper is an ion-exchange matrix with net negative charge at most pHs. At low pH (such as in 75 mM orthophosphoric acid, which is used to wash the paper in this protocol), the excess [γ-^{32}P]ATP left after a kinase assay will not bind. Under the same conditions, however, the phosphorylated peptide will bind to the P81 paper if it carries a net positive charge at low pH. In practice, this is usually achieved by tagging a peptide with two or three arginine or lysine residues at the N- or C-terminus. At the pH of 75 mM orthophosphoric acid the arginyl or lysyl side chains are positively charged and will bind to P81 paper. It is necessary, of course, that the activity of the kinase in question is not affected by the addition of basic residues to the substrate. Usually five or more residues are placed between the hydroxy-amino acid phosphorylation site and the basic residues required for binding to P81 paper.

When proteins are used as substrates for kinases, the net charge of the protein is also a consideration for adsorption to P81 paper. As for peptides, adsorption to P81 paper is more suitable for basic proteins than for acidic proteins. Under the conditions described in this protocol, histones bind very well to P81 paper because they are highly basic, but casein binds very poorly or not at all because it is a very acidic protein.

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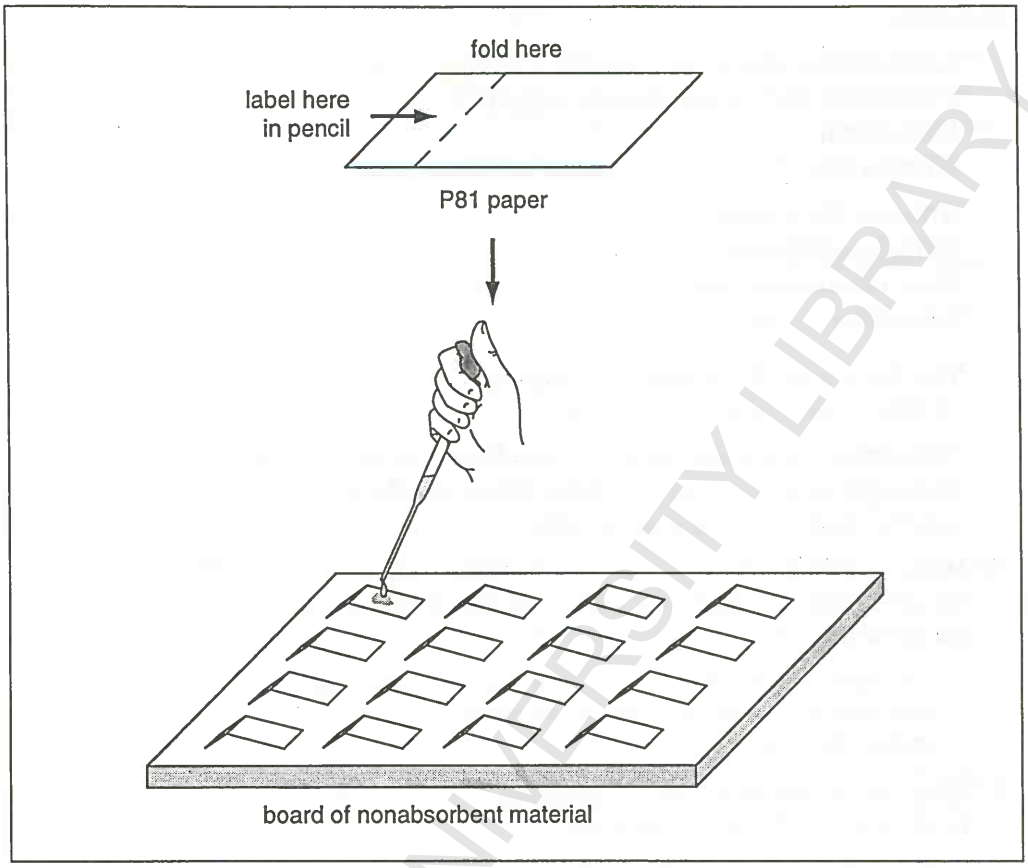

Figure 13.7.1 Addition of sample to P81 phosphocellulose paper. The 2 × 2–cm squares of P81 paper are labeled using a pencil (ink is dissolved by the acetone wash), and 50% to 75% of the reaction product is applied to the paper.

Materials

Assay samples (see Basic Protocols 1 to 5 and Alternate Protocol)
75 mM orthophosphoric acid (stored up to 6 months at room temperature)
Acetone

2 × 2–cm squares of P81 phosphocellulose paper (Whatman)
500-ml plastic beaker with the bottom replaced with solvent-resistant plastic or wire mesh (see Fig. 13.7.2)
20-ml scintillation vial
Scintillation counter

1. Cut 2 × 2–cm squares of P81 paper, fold the end over, and label the folded end with pencil as shown in Figure 13.7.1. Place each square on a large piece of nonabsorbent material, such as plastic or acrylic sheeting or an aluminum foil–covered fiberboard.

   Use a soft pencil to label the squares of P81 paper, as the final acetone wash will remove or destroy ink labels.

2. Pipet 15 or 30 µl of the contents of each assay tube quickly onto the surface of the prepared P81 paper squares. Immediately place the paper squares in a 500-ml plastic beaker with the bottom replaced with solvent-resistant plastic or wire mesh (Fig. 13.7.2).

   Usually 50% to 75% of the final reaction mixture is spotted onto PB1 paper to ensure that there is sufficient signal to be detected.

   If the plastic mesh used in the beaker is not solvent-resistant, the final acetone washes will dissolve it!

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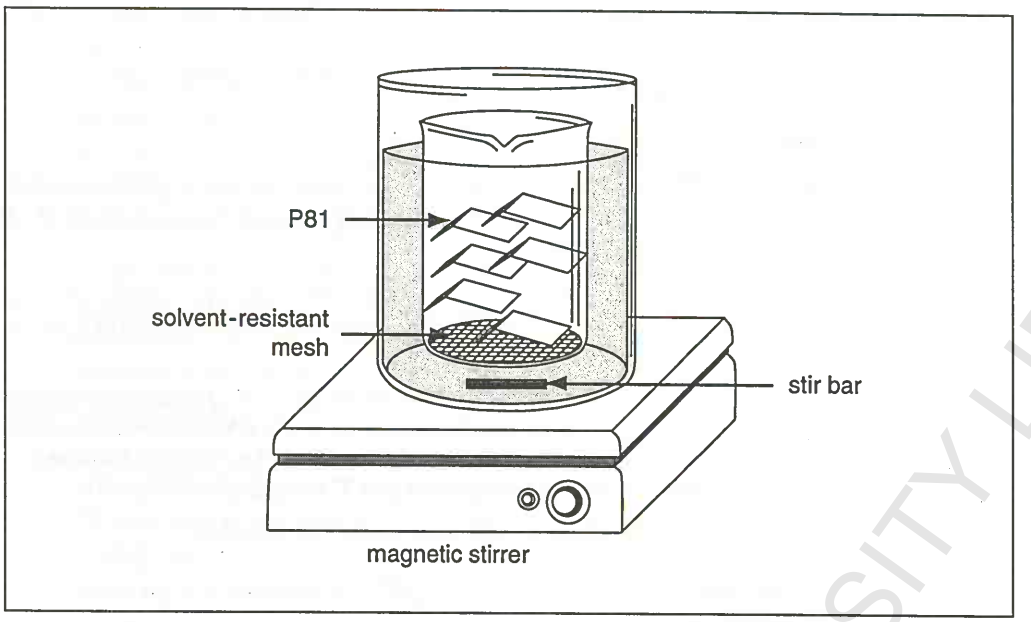

Figure 13.7.2 Setup for washing P81 phosphocellulose paper with reaction samples. The paper squares are washed five times for 5 min each in 75 mM orthophosphoric acid and once in acetone. They are then air dried and counted in a scintillation counter.

3. Place this beaker into a larger beaker containing 75 mM orthophosphoric acid and incubate 5 min with stirring.
4. Pour the used orthophosphoric acid wash into a container suitable for ^{32}P-containing liquid waste. Refill the large beaker with 75 mM orthophosphoric acid and incubate 5 min more. Repeat for a total of five 5-min washes.
5. Fill the large beaker with acetone and wash the P81 papers for 5 min. Discard the acetone and allow the papers to dry.
6. Place each square of P81 paper in a 20-ml scintillation vial and count by detection of Cerenkov radiation.

Alternatively, if the absolute dpm value is desired and a ^{32}P quenching curve is available for the scintillation counter, add scintillation fluid to the vials and count.

ELECTROPHORETIC ANALYSIS OF PHOSPHORYLATION

SUPPORT PROTOCOL 4

Electrophoretic analysis of phosphorylation allows a more specific determination of protein substrate phosphorylation, because other phosphoproteins are separated by electrophoresis. It is advantageous to use the resolving power of SDS-PAGE to assay relatively crude enzyme samples; this method of analysis may also give useful information on kinase autophosphorylation.

Stop the reaction (see Basic Protocols 1 to 5 or Alternate Protocol) by adding 20 µl ice-cold 2× SDS-PAGE sample buffer (*UNIT 10.1*). Mix thoroughly, heat the sample 3 min in a boiling water bath, then analyze by SDS-PAGE (*UNIT 10.1*). Stain, fix, dry, and autoradiograph the gel.

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REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

[γ-^{32}P]ATP solution

Dilute 10 mM Mg/ATP solution (see recipe) 1/10 in water to give a 1 mM solution. For the assay, add 10 µl of 1 mM Mg/ATP to 80 µl water and then add 10 µl of 10 mCi/ml [γ-^{32}P]ATP (3.3 pmol ATP).

The final ATP concentration is ~100 µM (plus 33 nM from the [γ-^{32}P]ATP, which is negligible), and the 100 µCi (~2.2 × 10^8 dpm) in 100 µl gives a specific activity of 1 µCi/µl or ~2.2 × 10^7 dpm/nmol.

Suppliers for [γ-^{32}P]ATP include Amersham, DuPont NEN, and ICN; it is usually available at a variety of specific activities. A good specific activity to use is 3000 Ci/mmol, normally provided by the manufacturer at a concentration of 10 mCi/ml. Amersham's Redivue is a convenient [γ-^{32}P]ATP solution to use as it is stored at 4°C instead of –20°C.

As 1 Ci is ~ 2.2 × 10^{12} dpm, then for a specific activity of 3000 Ci/mmol
= 3 Ci/µmol
= 6.6 × 10^{12} dpm/µmol
= 6.6 × 10^6 dpm/pmol
= 2.2 × 10^6 dpm ~ 1 µCi ~ 0.33 pmol.

The stock Mg/ATP solution prepared earlier can be diluted to a working ATP solution of the desired concentration (in this example, 0.1 mM).

CaM kinase reaction buffer, 5×

100 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)
25 mM $MgCl_2$
1 mM $CaCl_2$
Store up to 6 months at –20°C

Casein kinase reaction buffer, 5×

100 mM Tris·Cl, pH 7.5 at 30°C (*APPENDIX 2E*)
25 mM $MgCl_2$
2.5 mM DTT
750 mM KCl
Store up to 6 months at –20°C

Cyclic nucleotide–dependent protein kinase reaction buffer, 5×

250 mM Tris·Cl, pH 7.5 at 30°C (*APPENDIX 2E*)
25 mM $MgCl_2$
Store up to 6 months at –20°C

Lysis buffer

50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.4
100 mM NaCl
50 mM sodium fluoride
5 mM β-glycerophosphate
2 mM EDTA
2 mM EGTA
1 mM sodium vanadate (Na_3VO_4)
1% (v/v) Nonidet P-40 (NP-40) or Triton X-100
Store up to 6 months at –20°C

To stabilize kinase activity, specific kinase inhibitors, protease inhibitors, and reducing agents may be added to the lysis buffer (see Strategic Planning, Enzyme Sources).

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Mg/ATP solution, 10 mM

Stock solutions:

20 mM $MgCl_2$ or $MgSO_4$

20 mM Na_2ATP

Working solution: Mix equal volumes of the two stock solutions and measure the pH. Adjust the pH slowly to pH 7.4 with 100 mM HCl or 100 mM NaOH. Store up to 1 year in small aliquots at –20°C.

If a precipitate forms, the solution should be stirred continually and the pH maintained at 7.4.

Adenosine trisphosphate is normally purchased as a sodium salt. Most kinases require ATP to be complexed with magnesium for its efficient utilization as a substrate. In some cases manganese can substitute for Mg^{2+}.

PKC reaction buffer, 5×

Solvent:

100 mM Tris·Cl, pH 7.5 at 30°C (*APPENDIX 2E*)

25 mM $MgCl_2$

1 mM $CaCl_2$

Store up to 6 months at –20°C

Lipids:

Just before the buffer is to be used, transfer enough 5 µg/ml phosphatidylserine stock solution in chloroform to provide 100 µg phosphatidylserine/ml buffer (final concentration) to a clean tube. Add sufficient 0.5 µg/ml Diolein stock solution (Sigma) in chloroform to provide 10 µg Diolein/ml buffer (final concentration) to the same tube and mix. Dry the lipids under a stream of nitrogen.

Working solution:

Add the required volume solvent (see above) and place on ice for 10 min. Sonicate the mixture at medium power for 1 min while the sample is on ice. Let the sample sit on ice 2 min. Repeat sonication 4 more times for a total of 5 min. Discard any unused buffer.

Diolein is the old name for various preparations that contain mixed isomers of diacylglycerol, the cofactor for PKC. Diolein is a suitable source for the cofactor in these assays, and it is less expensive than pure preparations of 1,2-diacylglycerol.

Protease inhibitor stock solutions

100 mM phenylmethylsulfonyl fluoride (PMSF) in 100% ethanol

100 mM benzamidine

1 mg/ml leupeptin

1 mg/ml pepstatin A

1 mg/ml antipain

Store up to 6 months at –20°C

Synthetic peptide substrate solution

Prepare 10 mM solutions of each peptide (highest quality available) in Milli-Q water and adjust pH to 7.4 before storage at –70°C or use.

If the peptide is insoluble, check the sequence—a peptide with a high proportion of acidic residues may be more soluble at alkaline pH, and one with a high proportion of basic residues may be more soluble at acidic pH. Hydrophobic peptides may be solubilized by adding up to 20% (v/v) acetonitrile. Whatever is added must not compromise the assay conditions: e.g., if a peptide must be dissolved at pH 10, adding the solution to the reaction mix must not change the pH of the reaction.

Most synthetic peptides are quite stable when stored at –70°C; the stability of individual peptide substrates should be determined for the storage conditions used, however. If peptides are custom synthesized they should ideally be purified by HPLC before use.

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Tyrosine kinase reaction buffer, 5×

100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4
5 mM $MnCl_2$
5 mM DTT
500 µM sodium vanadate (Na_3VO_4)
Store up to 6 months at –20°C

COMMENTARY

Background Information

In recent years there has been an explosion of interest in protein kinases. It is now obvious that this class of enzyme plays many essential roles in a large array of biological processes, including the cellular responses to hormones, control of most stages of the cell cycle, and development of the nervous system in systems as diverse as fruit fly and man. Because of this interest, a very large number of protein kinase and protein phosphatase genes have been identified; current estimates are that there are well over 2000 genes encoding these enzymes in mammalian genomes. Obviously the study of protein kinases and phosphatases can provide fundamental information regarding the regulation of many diverse systems; the protocols provided in this unit include enough information to enable researchers new to this type of experiment to embark on the study of protein kinases.

Protein kinases can be assayed using two types of substrate, intact proteins or synthetic peptides, and there are advantages and disadvantages to the use of each. A number of intact proteins may be used as general protein kinase substrates, and those in common use are available in relatively pure form from a number of manufacturers at reasonable prices. The main disadvantage to the use of proteins as substrates is the lack of specificity, sometimes making interpretation of experimental data difficult. Intact proteins such as myelin basic protein (MBP) contain many different phosphorylation sites. MBP can be phosphorylated by MAP kinase, some protein kinase C isoforms, and Ca^{2+}/calmodulin kinase II, each at one or more sites. Hence, it is often impossible to differentiate which kinase is responsible for phosphorylating MBP in a complex mixture like a whole cell lysate. Some proteins cause headaches because they cannot be used with simple experimental protocols, e.g., casein.

The use of synthetic peptides improves the specificity of the reaction by providing a single, well-defined phosphorylation site. They also allow experiments to define the concensus sequence for phosphorylation by a particular protein kinase and make it possible to design a substrate that has excellent kinetic properties for the kinase of interest. Peptides are often easier to use than their protein counterparts as in the case of casein kinases, where peptides can be designed that allow the use of phosphocellulose paper rather than other more involved methods for assessment of experimental results. Use of peptides is not without disadvantages though, the most significant being the cost of producing reasonable quantities of large numbers of peptides. When this is coupled with the observation that sometimes peptides are not recognized by the kinase because some structural element is missing, using peptide substrates can be a frustrating and expensive business. However, this is the exception rather than the rule, and the use of specific peptides to assay protein kinases is ultimately the best choice.

One unit of kinase activity is usually defined as the amount of enzyme required to transfer 1 µmol of phosphate to a substrate from ATP per minute. From the following example it is possible to calculate a number of the reaction parameters:

$$\text{enzyme} + [\gamma\text{-}^{32}\text{P}]\text{ATP} + \text{substrate} \rightarrow \text{enzyme} + \text{ADP} + [^{32}\text{P}]\text{product}$$

For this example, 1 mg protein (based on protein assay; see *UNIT 3.4*) was used for the assay. The [γ-^{32}P]ATP in the assay mixture had a specific activity of 10^6 dpm/nmol. After the 10-min reaction the ^{32}P-labeled product had a specific activity of 10^5 dpm; therefore, there were ($10^5/10^6$) nmol of ^{32}P-labeled product, and the concentration was 10^{-1} nmol or 100 pmol. Hence, the enzyme transferred 10 pmol of phosphate per minute, and represents 0.01 U of enzyme activity when measured with this substrate. Put another way, this represents an enzyme specific activity of 10 pmol/min/mg of protein. This latter expression of enzyme activity is a useful measure when comparing different preparations of a kinase or, during purification of a kinase, as a measure of the extent of purification through different steps.

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Critical Parameters

Although it may seem obvious, the most critical element in performing kinase assays is using the correct controls. This is especially true when performing assays on tissue samples or cell extracts. Controls should always include addition of no substrate, addition of no enzyme source, addition of heat-denatured enzyme source, and, for reactions that require activator or cofactor, addition of any activator or cofactor and addition of no activator or cofactor.

Another important aspect is the concentration of ATP in the assay. The ATP concentration can vary depending on the type of experiment to be performed. For derivation of kinetic data, the concentrations of both ATP and peptide or protein substrate are critical. If kinase detection during purification is required, assay linearity is not as important as long as the right controls are present. For mapping in vitro phosphorylation sites on a kinase substrate, very often the ATP concentration is kept extremely low but the specific activity very high.

When designing potential peptide substrates for individual protein kinases, there are a number of considerations to remember. For example, phosphorylated peptide will bind to the P81 phosphocellulose paper. To be able to analyze the assay with this paper, it is necessary that the peptide carry a net positive charge at low pH. In practice this is usually achieved by tagging a peptide with two or three arginine or lysine residues at the N- or C-terminus. At the pH of 75 mM orthophosphoric acid, the arginyl or lysyl side chains are positively charged and will bind to the P81 paper. It is important to determine that the activity of the kinase of interest is relatively unaffected by the addition of basic residues to its peptide substrates in this way. To help ensure this is the case, it is a good idea to place five or more residues between the hydroxy-amino acid to be phosphorylated and the basic residues required for binding to P81 paper.

Net charge considerations also pertain to some extent to proteins used as substrates for protein kinases. Under the conditions described in Support Protocol 3, histones will bind very well to P81 paper, because they are highly basic, but casein, which is very acidic, binds very poorly or not at all. In these cases, the P81 paper assay is very good when histones are used to assay kinase activity, but worthless when casein is used.

When crude enzyme sources, such as cell lysates, are analyzed, many other proteins are present and phosphorylation of multiple proteins in the crude mixture is possible, as is autophosphorylation of the protein kinase. Because many proteins will be precipitated by trichloroacetic acid (TCA) or will bind to P81 paper, there can be elevated backgrounds and severe overestimates of peptide phosphorylation when Support Protocols 2 and 3 are used. When performing kinetic studies, this overestimation of phosphorylation can be very misleading, as substrates other than the peptide under study will undoubtedly be phosphorylated and with different kinetics.

Troubleshooting

Any troubleshooting of kinase assay procedures should be very straightforward. If the assay does not work, verify that both [γ-^{32}P]ATP and the peptide or protein substrate have been added at the correct concentrations. It is also very important to keep the enzyme source on ice before use and to store it appropriately between experiments. Very often when this type of experiment does not work it is because of a labile enzyme, poor storage of the enzyme, or a combination. It is important to know how long a particular enzyme is stable under the conditions used and the rate of inactivation or denaturation, if relevant. It is also essential to know if the enzyme activity is comparable between different preparations, especially for purified proteins.

Anticipated Results

Depending on the requirements, information can be obtained about the specific activity of a particular enzyme preparation, kinetic parameters for the reaction catalyzed, or whether a particular kinase has been identified or substantially purified.

Time Considerations

Kinase assays are quite simple to set up and perform. If the samples for assay are available, it is a matter of only ~3 to 4 hr to go from assay set up to data interpretation when either TCA precipitation (Support Protocol 2) or adsorption to phosphocellulose paper (Support Protocol 3) is used to separate phosphorylated protein from contaminating [γ-^{32}P]ATP. With SDS-PAGE analysis (Support Protocol 4), the assays can be set up, performed, and the samples run on a SDS-PAGE gel in ~4 to 5 hr. Gel drying and autoradiography can take from 2 hr to a number of days depending on the level of protein phosphorylation. In general, if individual protein bands have ~20,000 cpm of ^{32}P associated with them, they will be visible with 1 hr of autoradiography.

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Permeabilization Strategies to Study Protein Phosphorylation

This unit deals with the use of nucleotide triphosphates to label proteins in vitro in permeabilized cells and isolated cellular fractions. These experiments generally utilize [γ-^{32}P]ATP as an exogenously added phosphate donor, although [γ-^{32}P]GTP can be used in specific cases. The method is very straightforward, although numerous considerations must be made before applying it to each new system.

UNIT 13.2 describes labeling of phosphoproteins in intact cells with ^{32}P-labeled inorganic phosphate ($^{32}P_i$). After ascertaining that the protein of interest is a phosphoprotein using the protocols detailed in that unit, the phosphorylation event itself may be studied either in cell-free systems or in a more intact cellular system. In vitro, most protein kinases are capable of phosphorylating many substrates in addition to those phosphorylated in vivo, thus making it desirable to identify and study a protein kinase–catalyzed phosphorylation reaction in a more intact system than a cell lysate or homogenate. Permeabilization of intact cells provides a powerful experimental approach for accomplishing this. Brief permeabilization of cells is possible using a variety of agents. This procedure gives access to the intracellular milieu but still allows signal transduction events to be initiated by hormone stimulation of the cells via cell-surface receptors.

Brief chemical permeabilization of cells allows the introduction of cell-impermeable reagents into the intracellular compartment in order to study particular signal-transduction processes. In this type of experiment, relatively large molecules such as the Fab′ fragments of specific antibodies may be introduced into the permeabilized cell, although the kinetics of entry may be too slow for the study of some processes. This technique is often more useful when introducing relatively small molecules into the permeabilized cell, for example non-cell-permeable activators (e.g., hydrophilic or lipophobic small molecules that cannot partition into the membrane) or inhibitors of protein kinases or phosphatases (see, e.g., Calbiochem catalog). The kinetics of entry into permeabilized cells for small molecules such as [γ-^{32}P]ATP are fast, and the system equilibrates the cellular ATP and [γ-^{32}P]ATP with intracellular processes within 2 to 3 min. Figure 13.8.1 shows the

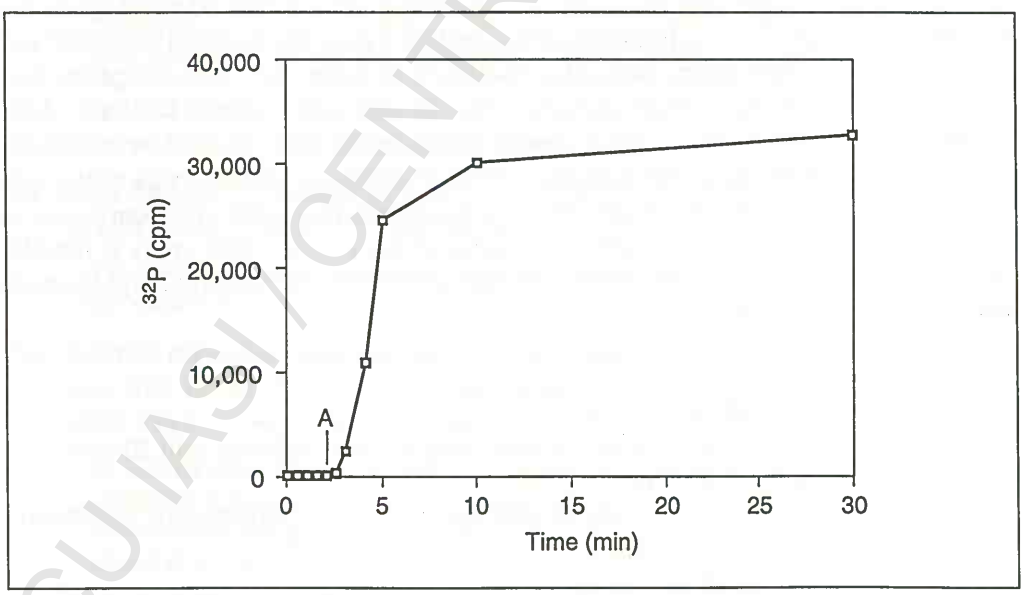

Figure 13.8.1 Equilibration of the monoester phosphates of the lipid phosphatidylinositol-4,5-bisphosphate with the ATP pool after permeabilization of human platelets. The letter A marks the point of addition of [γ-^{32}P]ATP.

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equilibration of the monoester phosphates of the lipid phosphatidylinositol-4,5-bisphosphate with the ATP pool after permeabilization of human platelets. As these monoester phosphates are known to equilibrate with the ATP pool extremely quickly, this experiment provides a good indication of the speed of equilibration of cellular ATP pools with intracellular processes after addition of [γ-^{32}P]ATP to permeabilized cells.

Procedures are outlined for performing a protein phosphorylation experiment using permeabilized cells (see Basic Protocol 1) and isolated intracellular organelles (see Basic Protocol 2). Both of these procedures result in lysates from which the protein of interest may be easily immunoprecipitated; however alternative techniques are described for preparing the final lysate from either Basic Protocol 1 or Basic Protocol 2 for electrophoretic analysis (see Alternate Protocols 1, 2, 3, and 4). A related procedure that does not involve permeabilization is outlined for direct analysis of cytosolic or membrane-bound kinases (see Alternate Protocol 5). Two different methods for determining the specific radioactivity of ^{32}P-containing compounds are also included (see Support Protocols 1 and 2).

CAUTION: It cannot be emphasized enough that each investigator should be fully familiarized with their local regulations regarding the safe handling and disposal of ^{32}P and also have the appropriate equipment for dealing with the containment of ^{32}P. See *APPENDIX 2B* for more information on the safe use of radioisotopes.

NOTE: Milli-Q purified water or its equivalent should be used to prepare all solutions used throughout this unit.

BASIC PROTOCOL 1

ANALYSIS OF PROTEIN PHOSPHORYLATION IN PERMEABILIZED CELLS

When setting out to study a protein phosphorylation event via a permeabilization protocol, the general procedure is as follows. First, cells are incubated in a buffer which has been designed to mimic the composition of the intracellular compartment and which contains a permeabilizing agent. The preferred permeabilization agent of choice is streptolysin O, as this agent allows many signal-transduction systems to remain intact for a number of minutes after its addition (Stephens et al., 1994). This is certainly not true of all permeabilization agents or protocols; if another agent (e.g., saponin) is to be tried, the methods detailed below should be used as a general guideline only. Depending on the experiment to be performed, the permeabilization buffer may also contain [γ-^{32}P]ATP and the particular reagents under study. The [γ-^{32}P]ATP provides the "hot" phosphate, and will equilibrate with the intracellular processes utilizing ATP within 2 to 3 min (A.N. Carter, unpub. observ.). After a brief time the experimental reagents, such as inhibitors, can be introduced into the permeabilization medium and hence into the inside of the cell. At this point the cells may be stimulated with the appropriate agent in the same way as for intact cell preparations. The phosphorylation of the protein under study is initially investigated by immunoprecipitation as described in *UNIT 13.2*, followed by phosphopeptide mapping as described in *UNIT 13.3*.

Materials

Cell culture to be labeled
Cell culture medium appropriate for cells being studied, buffered with 20 mM HEPES, pH 7.2, prewarmed to 37°C
Cells under study (normally cultured cell lines or isolated primary cells in culture)
Extracellular buffer (see recipe), 37°C
Permeabilization buffer (see recipe), 37°C
60 U/ml streptolysin O working solution (see recipe), freshly prepared
10 mM cold ATP stock solution (see recipe)
10 mCi/ml [γ-^{32}P]ATP (3000 Ci/mmol; e.g., DuPont NEN)

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Pharmacological agents, peptides, or Fab′ fragments to be studied
Appropriate receptor agonists
2× lysis buffer for immunoprecipitation (see recipe), ice-cold
Protease inhibitor stock solutions in absolute ethanol (store up to 6 months at –20°C):
  100 mM phenylmethylsulfonyl fluoride (PMSF)
  100 mM benzamidine
  1 mg/ml pepstatin A
  1 mg/ml leupeptin
  1 mg/ml antipain
100 mM DTT
Dry ice/ethanol bath

Noncirculating 37°C water bath containing a flat shelf (preferably made of wire mesh) with space for the number of cell plates in the experiment
50-ml centrifuge tubes
Tabletop centrifuge
Cell scrapers
Screw-cap microcentrifuge tubes

Permeabilize cells

For monolayer cultures

1a. Approximately 1 hr before starting the experiment, change the medium in the cell culture plates to be labeled to equivalent medium containing 20 mM HEPES. Return cells to incubator and allow the cells to equilibrate with the new medium before use.

The number of cells used for any experiment of this type is totally dependent on how easy it is to detect the end product being studied. In some cases, a 60-mm dish of cells that has just reached confluency, with ~1 × 10^6 cells, is adequate; in others a 100-mm dish of cells with ~1 × 10^7 cells is required. It is advisable to start with ~1 × 10^6 cells and see if the signal is detectable. Use of a 60-mm dish allows a smaller volume of permeabilization medium (normally of the order of 0.5 to 2 ml) to be used.

Standard cell culture medium is buffered by bicarbonate ions in the medium and the CO_2 in the atmosphere of the incubator. Outside the incubator this buffering system is ineffective and the pH will quickly rise if the culture is left in the normal atmosphere. The pH of HEPES buffers are minimally affected by temperature (unlike Tris buffers), hence they provide good pH control for cell culture medium. One drawback is that HEPES may be toxic to some cells above a concentration of ~20 mM, so it is important to check possible cellular toxicity before using HEPES-buffered culture media.

2a. Place the required number of plates containing cells in the 37°C water bath and allow 10 min for the medium to reequilibrate.

The cells are stable in this state for an hour or so, depending on the rate of evaporation of the culture medium.

3a. Aspirate the culture medium from each plate and quickly rinse the cells twice, each time with 10 ml of 37°C extracellular buffer. Aspirate the extracellular buffer, then add 0.5 to 2 ml permeabilization buffer to each plate.

Be careful when rinsing, as some cell lines are less adherent than others. Usually, most epithelial or mesenchymal lines adhere well and will not slough off the dish unless extreme force is used. Transformed cells usually adhere poorly and can easily be rinsed off the dish if care is not taken.

4a. Add streptolysin O (from the 60 U/ml working solution) to each plate to a final concentration of 0.6 U/ml. Add cold ATP (from the 10 mM stock solution) to a final concentration of 100 μM.

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For suspension cultures

1b. Approximately 1 hr before starting the experiment, transfer cells to sterile 50-ml centrifuge tubes and centrifuge 5 min at 1000 × *g*, room temperature. Remove the supernatant, then rinse cells twice, each time by adding ~40 ml of 37°C extracellular buffer, centrifuging 5 min at 1000 × *g*, room temperature, and removing the supernatant.

The cell suspension may be split into aliquots, if desired, after the addition of the final rinse of extracellular medium. To do this, add the final (40-ml) rinse of extracellular medium, resuspend cells gently, and dispense aliquots into fresh tubes. Centrifuge the aliquots and remove the supernatants to complete the final rinse before proceeding to step 2b.

2b. Resuspend the cells in 37°C extracellular buffer at a density of 1×10^7 cells/ml. Allow the cells to equilibrate 15 to 30 min at 37°C with gentle swirling.

This step may have to be modified if a cell is used that could be activated by this procedure, (e.g., platelets). If a sensitive cell is used, equilibration time may be reduced to 5 to 10 min and the agitation may be omitted.

3b. Centrifuge cells again for 5 min at 1000 × *g*, room temperature, and aspirate the buffer.

4b. Add streptolysin O (from the 60 U/ml working solution) to fresh 37°C permeabilization buffer to a final concentration of 0.6 U/ml. Add cold ATP (from the 10 mM stock solution) to a final concentration of 100 µM. Add this permeabilization solution containing streptolysin O and ATP to the cells and mix gently but thoroughly.

Addition of cold ATP at the same time as streptolysin O allows cellular ATP levels to be maintained and will therefore preserve cell viability. One important consideration is whether the cells under study possess cell-surface receptors for ATP. The ATP acts as a full agonist for some types of purinergic receptors, and this can obviously cause confusion when analyzing signal-transduction mechanisms. Prior to performing permeabilization studies, first determine, using intact cells, whether extracellular ATP activates signal-transduction mechanisms that interfere with the system under investigation. Stephens et al. (1994) have developed a way of inactivating extracellular receptors for ATP in neutrophils prior to permeabilization. The utility of this method for other cell types is not known to date, although it may be used as a guideline if required. Readers are referred to the original paper for full discussions of this method.

Permeabilize and label cells

5. Permeabilize cells by incubating 1 to 5 min at 37°C.

The time of incubation depends very much on the particular system under study and the cell type, and must be determined empirically. Permeabilization for >5 min usually reduces hormone responsiveness of cells dramatically. This is probably due to a number of processes, including the loss of cytosolic components into the extracellular pearmeabilization buffer and physical uncoupling of receptors from signal-transduction processes.

6. Add 10 mCi/ml [γ-^{32}P]ATP to a final concentration of 50 to 500 µCi/ml.

[γ-^{32}P]ATP is usually purchased at a specific activity of ~3000 Ci/mmol, and a concentration of 10 mCi/ml. Hence 5 µl of the stock is 50 µCi [γ-^{32}P]ATP. The label may be used at this concentration. If the stock [γ-^{32}P]ATP is to be diluted into another solution it is better to do this directly before use.

The criterion for the amount of radiolabeled ATP to add (between 50 and 500 µCi/ml) is the detection limit. Initial experiments must be performed to see how much label must be added.

The alternative to this separate labeling step is to add the [γ-^{32}P]ATP at the same time as the streptolysin O and cold ATP in step 4a or b. The type of experiment to be performed will determine at which point the [γ-^{32}P]ATP is added to the cells.

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7. Add the pharmacological agents, peptides, or Fab′ fragments under study and allow time for their action as required.

Many pharmacological agents amenable to testing by this protocol are listed in the Calbiochem catalog. For example, a peptide containing residues 280 to 305 of the CaM kinase II α subunit can be a potent inhibitor of CaM kinase II and is non-cell-permeable. There are other peptides modeled on pseudosubstrate inhibitors of a variety of protein kinases that can be used as specific inhibitors, all of which are non-cell-permeable. Any Fab′ fragment may be tested by this protocol as long as it has an effect on enzyme activity, either positive or negative. Similarly, if an antibody stops an enzyme from reaching its site of action, it may also be tested via this procedure.

The time for a particular agent to act as required should be determined experimentally as part of the initial experimental protocols. As with the [γ-^{32}P]ATP addition, these agents may also be added at the same time as streptolysin O and cold ATP.

8. Add receptor agonists as required.

The same considerations are true for receptor agonist additions as for inhibitor and [γ-^{32}P]ATP additions to the cells. One must perform preliminary experiments to empirically determine the timings for these additions for each cell type under study.

The remaining steps of this protocol are to prepare the cells for analysis by immunoprecipitation. If electrophoretic analysis is to be performed, see Alternate Protocols 1 and 2.

Prepare cell lysates for immunoprecipitation

9a. *For monolayer cultures:* Stop the reactions by rapidly aspirating the permeabilization buffer and adding 0.75 ml ice-cold 2× lysis buffer for immunoprecipitation to each plate. Add protease inhibitors and DTT (as stock solutions) to the following final concentrations:

PMSF to 1 mM final
Benzamidine to 1 mM
Pepstatin A to 5 μg/ml final
Leupeptin to 5 μg/ml final
Antipain to 5 μg/ml final
DTT to 1 mM final.

Place plates on ice for 10 min, then scrape the cell debris from the bottoms of the plates into the buffer using a separate cell scraper for each plate.

9b. *For suspension cultures:* Stop the reactions by adding an equal volume of ice-cold 2× lysis buffer for immunoprecipitation. Add protease inhibitors and DTT to the final concentrations in step 9a, then place the tubes of cells on ice for 10 min.

10. Transfer lysates to screw-cap microcentrifuge tubes (one plate or cell aliquot/tube). Microcentrifuge lysates at maximum speed, 4°C, then transfer the supernatants to fresh screw-cap microcentrifuge tubes and freeze immediately in a dry ice/ethanol bath. Store at –70°C until needed.

The lysates are now ready for immunoprecipitation.

CAUTION: *The permeabilization buffer in the latter steps of this protocol contains [γ-^{32}P]ATP, and great care should be taken to remove the medium safely and dispose of it correctly as liquid radioactive waste. The lysates and cell scrapers are also very radioactive at this point. Screw-cap tubes should be used at all subsequent stages of processing the cell lysates as these provide the best containment of the ^{32}P-containing samples. The same microcentrifuge should be used for all centrifugation steps; after use it will undoubtedly be contaminated with ^{32}P. It can then be set aside for future use with ^{32}P-containing samples with the appropriate safety precautions.*

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INTACT CELL SAMPLE PREPARATION FOR ELECTROPHORETIC ANALYSIS OF PROTEIN PHOSPHORYLATION

The preceding protocol (see Basic Protocol 1) is set up to allow immunoprecipitation of specific proteins after the permeabilization experiment. Two alternatives to this approach, described below, involve using the separating power of polyacrylamide gel electrophoresis (PAGE) to fractionate proteins, instead of immunoprecipitation. Fractionation can be either by one-dimensional SDS-PAGE (*UNIT 10.1*), or by two-dimensional PAGE in which isoelectric focusing is performed as the first dimension, followed by SDS-PAGE (*UNIT 10.4*). In each case, sample loading must be optimized so that best resolution of the protein of interest is achieved.

These protocols are written for both monolayer cells and suspension cells. To perform the procedure with suspension cells, the cells must be centrifuged down as quickly as possible after reacting with the appropriate pharmacological agents/receptor agonists, then treated as described for monolayer cultures. Obviously, the timing of the reactions is less precise this way, but experiments can be done successfully and reproducibly if reaction and centrifugation times are kept exactly the same.

CAUTION: Samples for SDS-PAGE or isoelectric focusing will still contain a great deal ^{32}P at the latter stages of the protocol. Great care should be exercised in sample preparation. In particular do not use a probe sonicator to shear DNA, as ^{32}P-containing aerosols will be produced. In the case of SDS-PAGE, the gels should be run until the dye front passes out of the gel. This means that the SDS-PAGE running buffer will be also be contaminated with ^{32}P. In the case of isoelectric focusing the ^{32}P will focus out of the gel during electrophoresis; hence the running buffer will be contaminated with ^{32}P. These solutions should be disposed of as radioactive waste as local regulations dictate.

ALTERNATE PROTOCOL 1

Intact Cell Sample Preparation for SDS-PAGE

Additional Materials (also see Basic Protocol 1)

2× SDS-PAGE sample buffer (see recipe), 4°C
Bath sonicator
Boiling water bath

1. Prepare cells and perform permeabilization and labeling (see Basic Protocol 1, steps 1 through 8).

2a. *For monolayer cultures:* Stop the reactions by rapidly aspirating the permeabilization buffer and adding 100 to 250 µl of 4°C 2× SDS-PAGE sample buffer to each plate. Quickly scrape the cells into the buffer using a separate cell scraper for each plate and quantitatively transfer the sample to a screw-cap microcentrifuge tube.

2b. *For suspension cultures:* Microcentrifuge the reaction tubes 1 min at maximum speed, room temperature. Quickly aspirate the supernatants, taking care not to disturb the cell pellets. Add 100 to 250 µl of 2× SDS-PAGE sample buffer to each tube and vortex thoroughly.

When performing this step for suspension cultures it is very important to use exactly the same times for centrifugations if reproducible data are to be obtained.

3. If samples are very viscous as a result of release of DNA from cell nuclei, shear the DNA by placing the capped sample tubes in a bath sonicator filled with ice water and sonicating 5 min.

4. Boil samples 3 min in a boiling water bath.

To perform the analysis, carefully apply up to 50 µl of the samples to the sample wells of a precast SDS-PAGE gel. Proceed as described in UNIT 10.1.

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Intact Cell Sample Preparation for Isoelectric Focusing

ALTERNATE PROTOCOL 2

Additional Materials (also see Basic Protocol 1)

Two-dimensional-PAGE lysis buffer (see recipe), 4°C
Two-dimensional-PAGE sample buffer (see recipe)

Bath sonicator
Dry ice/ethanol bath
Lyophilizer

1. Prepare cells and perform permeabilization and labeling (see Basic Protocol 1, steps 1 through 8).

2a. *For monolayer cultures:* Stop the reactions by rapidly aspirating the permeabilization buffer and adding 100 to 250 µl of 4°C two-dimensional-PAGE lysis buffer. Scrape the cells into the buffer using a separate cell scraper for each plate and transfer the sample to a screw-cap microcentrifuge tube.

2b. *For suspension cultures:* Microcentrifuge the reaction tubes 1 min at maximum speed, room temperature. Quickly aspirate the supernatant, taking care not to disturb the cell pellet. Add 100 to 250 µl of two-dimensional-PAGE lysis buffer to each tube and vortex thoroughly.

When performing this step for suspension cultures it is very important to use exactly the same times for centrifugations if reproducible data are to be obtained.

3. If samples are very viscous as a result of release of DNA from cell nuclei, shear the DNA by placing the samples in a bath sonicator filled with ice water and sonicating 5 min. Freeze samples in a dry ice/ethanol bath, then lyophilize.

4. Redissolve the lyophilized samples in 50 to 100 ml of two-dimensional-PAGE sample buffer. Warm the samples to 37°C for 3 to 5 min.

To perform the analysis, apply 10 to 20 µl of each sample to a prepared isoelectric focusing gel of the required pH gradient (see UNITS 10.2 & 10.4). Samples for isoelectric focusing must have a low salt concentration because a high salt concentration often causes band broadening during isolectric focusing.

ANALYSIS OF PROTEIN PHOSPHORYLATION USING ISOLATED SUBCELLULAR FRACTIONS

BASIC PROTOCOL 2

When the protein under study exists in a particular organelle and is phosphorylated in intact cells, one consideration when studying the phosphorylation event in vitro in isolated organelles is whether the protein is accessible to exogenously added kinases or phosphatases, or whether it is accessible to endogenous kinases or phosphatases. For instance, if it is a protein of the endoplasmic reticulum, is its location transmembrane or intraluminal? If transmembrane, is it normally phosphorylated on the cytosolic side or luminal side of the membrane?

Permeabilization protocols can also be used with intact intracellular organelles as well as intact cells. This is obviously an important consideration when dealing with proteins found inside the organelle in question. Another consideration here is whether the organelle can be isolated in an intact condition, or whether its contents will be partially or completely lost during purification. In the case of mitochondria this does not pose a problem, but for more fragile organelles such as the Golgi apparatus this may be more problematic.

The study of protein phosphorylation in isolated organelles (*UNIT 4.2*) can be a straightforward extension of the permeabilization protocols described above (see Basic Protocol 1).

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The isolated organelle is treated in the same way as one would treat cultured cells in suspension. However, as there is little or no requirement for the maintenance of signal-transduction mechanisms in isolated organelles, and the permeabilization procedure can be more prolonged than for intact cells. This allows the introduction of larger molecules such as purified protein kinases into the organelles under study.

Materials

Suspension of isolated intracellular organelles (*UNIT 4.2*)
Intracellular buffer (see recipe), 4°C
Permeabilization buffer (see recipe), 37°C
60 U/ml streptolysin O working solution (see recipe)
10 mM cold ATP stock solution (see recipe)
10 mCi/ml [γ-^{32}P]ATP (3000 Ci/mmol; e.g., DuPont NEN)
Reagents under study: e.g., kinase/phosphatase inhibitors, protein kinases, or phosphatases
2× lysis buffer for immunoprecipitation (see recipe), 4°C

Screw-cap microcentrifuge tubes
Benchtop ultracentrifuge (e.g., Beckman TL-100 or Airfuge)
37°C circulating water bath

1. Wash the isolated intracellular organelle preparation three times with 4°C intracellular buffer, each time by ultracentrifuging 10 min at ≥100,000 × *g*. Transfer the equivalent of 1 mg protein to individual screw-cap microcentrifuge tubes and keep on ice.

   The best way to wash the organelle preparations is to use a Beckman TL-100 benchtop ultracentrifuge or the older Beckman Airfuge. Both of these instruments will produce g-forces >100,000 × g. Because of the difficulty in centrifuging some organelles (e.g., plasma membrane preparations), it is essential to use ultracentrifugation for all organelles except nuclei, which can be recovered by microcentrifuging 30 sec at maximum speed. See UNIT 4.2 for details of organelle isolation.

2. Ultracentrifuge the organelle preparation 10 min at ≥100,000 × *g*, 4°C, and aspirate the supernatant. Add 200 ml 37°C permeabilization buffer, mix gently, and transfer to the 37°C circulating water bath.

   Work quickly at this point to minimize any proteolytic and other unwanted side reactions.

3. Add streptolysin O (from the 60 U/ml working solution) to each tube to a final concentration of 0.6 U/ml. Add cold ATP (from the 10 mM stock solution) to a final concentration of 100 µM.

   Exactly the same considerations apply when performing permeabilization of organelles as when performing permeabilization of intact cells (see Basic Protocol 1). However, because there is no intact signal-transduction machinery in isolated organelle preparations, the range of possible experiments is fewer, so the system is usually more robust than for intact-cell experiments.

4. Add 10 mCi/ml [γ-^{32}P]ATP to a final concentration of 50 to 500 µCi/ml.

   The criterion for the amount of radiolabeled ATP to add (between 50 and 500 µCi/ml) is the detection limit. Initial experiments must be performed to see how much label must be added.

5. Add the reagents under study—e.g., kinase/phosphatase inhibitors, protein kinases, or phosphatases.

6. Add 200 ml of 4°C 2× lysis buffer to each tube to lyse the organelles. Mix well and place on ice for 5 min.

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7. Microcentrifuge the samples 10 min at maximum speed, 4°C. Store lysates at –70°C.

The lysates are now ready for immunoprecipitation.

ORGANELLE SAMPLE PREPARATION FOR ELECTROPHORETIC ANALYSIS OF PROTEIN PHOSPHORYLATION

Exactly the same considerations apply to the electrophoretic analysis of protein phosphorylation in isolated organelles as apply to permeabilized cells (see Alternate Protocols 1 and 2). Organelle preparations must first be ultracentrifuged and the supernatant removed before adding the appropriate buffer for electrophoresis. For this, the use of a Beckman TL-100 benchtop ultracentrifuge, which can provide *g*-forces >300,000 × *g*, is recommended. Complete removal of the supernatant is especially important if isoelectric focusing for two-dimensional-PAGE (Alternate Protocol 4) is used, as the salt concentration must be kept low.

CAUTION: Samples for SDS-PAGE or isoelectric focusing will contain a large amount of ^{32}P at the latter stages of the protocol. Great care should be exercised in sample preparation. In the case of SDS-PAGE, the gels should be run until the dye front passes out of the gel. This means that the SDS-PAGE running buffer will be also be contaminated with ^{32}P. In the case of isoelectric focusing, the ^{32}P will focus out of the gel during electrophoresis; hence the running buffer will be contaminated with ^{32}P. These solutions should be disposed of as radioactive waste as local regulations dictate.

Organelle Sample Preparation for SDS-PAGE

ALTERNATE PROTOCOL 3

Additional Materials (also see Basic Protocol 2)

2× SDS-PAGE sample buffer (see recipe)
Boiling water bath

1. Prepare organelles and perform permeabilization and labeling (see Basic Protocol 2, steps 1 through 5).
2. Ultracentrifuge samples 10 min at 100,000 × *g*, 4°C, and aspirate the supernatant. Add 50 µl of 2× SDS-PAGE sample buffer and mix well.
3. Boil the samples 3 min in a boiling water bath.

The samples are now ready for analysis by SDS-PAGE (UNIT 10.1).

Organelle Sample Preparation for Isoelectric Focusing

ALTERNATE PROTOCOL 4

Additional Materials (also see Basic Protocol 2)

Two-dimensional-PAGE lysis buffer (see recipe)
Lyophilizer

1. Prepare organelles and perform permeabilization and labeling (see Basic Protocol 2, steps 1 through 5).
2. Ultracentrifuge samples 10 min at >100,000 × *g*, 4°C, and aspirate the supernatant. Add 200 µl of two-dimensional-PAGE lysis buffer and mix well.
3. Lyophilize the samples overnight.

The samples are now ready for analysis by isoelectric focusing (UNITS 10.2 & 10.4). Samples for isoelectric focusing must have a low salt concentration because a high salt concentration often causes band broadening during the isoelectric focusing dimension.

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ALTERNATE PROTOCOL 5

DIRECT ANALYSIS OF CYTOSOLIC OR MEMBRANE-BOUND KINASES

This protocol utilizes isolated subcellular fractions in straightforward kinase assays by adding exogenous, purified protein kinases to the reaction mixture containing the organelle in question. This works only if the protein under study is phosphorylated by a cytosolic or membrane-bound kinase and its substrate is on the cytosolic face of the organelle. One can then determine the phosphorylation of proteins by analyzing the reaction mixture by SDS-PAGE. It is a good idea to perform the reactions and subsequent manipulations in screw-cap microcentrifuge tubes to minimize the risk of ^{32}P contamination.

This protocol may be used to extend findings based on the use of the permeabilization strategies and assays with isolated organelles (see Basic Protocol 2). Hence, if a protein of interest is phosphorylated, it can be useful to determine the subcellular localizations of the kinase(s) responsible, as this may make it possible to infer clues as to the identity of the kinase(s).

Materials

Appropriate kinase assay buffer (*UNIT 13.7*), 4°C
[γ-^{32}P]ATP solution (see recipe in *UNIT 13.7*)
Suspension of isolated intracellular organelles (*UNIT 4.2*), 4°C
Purified protein kinase of interest (keep on ice)
2× SDS-PAGE sample buffer (see recipe), ice-cold

Screw-cap microcentrifuge tubes
30°C and boiling water baths

1. Prepare the following reaction mixture in a screw-cap microcentrifuge tube at 4°C:

   10 µl 5× kinase assay buffer
   5 µl [γ-^{32}P]ATP solution (5 µCi/5 µM ATP final)
   1 to 35 µl isolated intracellular organelle suspension (i.e., volume equivalent to 1 mg protein)
   H_2O to 50 to 100 µl.

   After reaction mix has been assembled, warm to 30°C for 5 min.

2. Start reaction by adding the purified protein kinase of interest. Incubate 15 min at 30°C.

   In the case of immunoprecipitated enzymes adsorbed onto beads, the assay mix minus the enzyme source can be made up complete first. This can then be dispensed, prewarmed, onto the immunoprecipitates.

   Incubation time can be adjusted as necessary depending on the extent of protein phosphorylation observed and the linearity of the reaction.

3. Stop the reactions by the addition of 100 µl of ice-cold 2× SDS-PAGE sample buffer. After thorough mixing, heat the samples 3 min in a boiling water bath.

   The samples are then ready for resolution by SDS-PAGE (UNIT 10.1).

   Samples for SDS-PAGE contain all the ^{32}P originally in the assays. Great care should be exercised in sample preparation and loading. The gels should be run until the dye front passes out of the gel. This means that the SDS-PAGE running buffer will be contaminated with ^{32}P, and this should be disposed of as radioactive waste as local regulations dictate.

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DETERMINATION OF SPECIFIC RADIOACTIVITY

The specific radioactivity (specific activity) of any radioactive compound is defined as the number of atoms of the radioactive isotope in a molecule as a proportion of the total number of atoms of the same type. The structure of ATP is shown in Figure 13.8.2, and the form used in the study of protein kinases is [γ-^{32}P]ATP, indicating that only the γ phosphate group has the radioactive ^{32}P atom. There are, however, two other atoms of ^{31}P. Hence, to attain the maximum possible specific activity, one should replace 100% of all phosphorus atoms with the ^{32}P isotope. This is obviously not necessary in the case of kinase phosphotransfer reactions, as only the γ phosphate is transferred to a substrate; therefore in practice it is irrelevant whether the α and β phosphate groups have the ^{32}P label. The units used by vendors of [γ-^{32}P]ATP are curies per mole (Ci/mol), even though the curie is no longer the international unit of radioactivity.

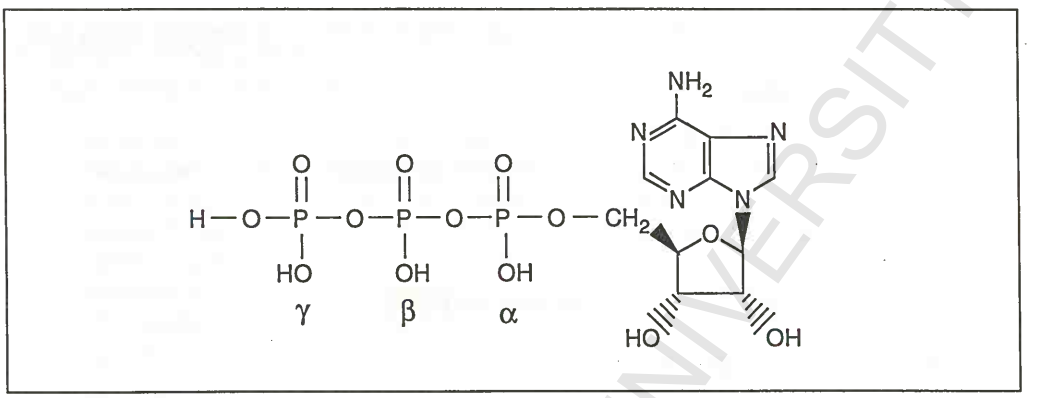

Figure 13.8.2 Structure of ATP highlighting the α, β, and γ phosphates/phosphorus atoms.

It is important to know the specific radioactivity of the ATP in a kinase assay, as important data can be derived from this information. Consequently this can be determined as required, although one can make assumptions based on the volume of [γ-^{32}P]ATP added to cold ATP prior to the assay. Obviously, the specific activity of [γ-^{32}P]ATP cannot be assumed when adding [γ-^{32}P]ATP to permeabilized cells or after labeling intact cells with $^{32}P_i$. In these cases, one can determine the specific activity experimentally as described below.

The specific radioactivity of [γ-^{32}P]ATP can be determined by a number of methods, but the following are reliable and relatively simple. Methodologies for the extraction of ATP from either $^{32}P_i$-labeled cells or from cell lysates are detailed below (Stephens and Downes, 1990). The basis for the extraction is the same in each case, but slightly different alternative steps are required. Protein or cell debris is first precipitated with perchloric acid and removed by centrifugation. The supernatants are then neutralized with a mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane (Freon). The aqueous phase from this step can then be directly analyzed for ATP concentration and ^{32}P content. ATP may be quantified using anion-exchange HPLC to separate nucleotides and a UV detector to detect nucleotides via UV absorption at 254 nm. The peak areas associated with injection of different amounts of ATP onto the column are then calculated and a standard curve constructed. The radioactivity associated with the ATP peak is easily determined by scintillation counting.

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Materials

^{32}P-labeled cells or cell lysates
Phosphate-buffered saline (PBS; *APPENDIX 2E*)
Standard ATP samples of known concentration in 4% perchloric acid
4% or 8% (v/v) perchloric acid, ice-cold
Tri-*n*-octylamine (Sigma)
1,1,2-trichlorotrifluoroethane (Freon; e.g., Aldrich, Sigma)
Linear gradient consisting of:
  Solution A: Milli-Q water filtered through 0.2-mm filter
  Solution B: 1.25 M $NH_4H_2PO_4$, pH 3.8 (adjust pH with H_3PO_4; filter through 0.2-mm filter)
Nucleotide standard mix: e.g., 1 mM each of AMP, ADP, ATP, GMP, GDP, and GTP

Cell scrapers
Polypropylene screw-cap microcentrifuge tubes or 5-ml polypropylene tubes with very tight-fitting caps
Tabletop centrifuge
HPLC system consisting of:
  Whatman Partisphere SAX HPLC column and guard column
  Apparatus for producing linear gradient
  UV detector set at 254 nm
  Fraction collector
Whatman low-dead-volume 0.2-mm syringe filters

Extract ATP from cells or cell lysates

For labeled cells

1a. Aspirate the medium from the labeled cells and rinse twice with PBS. Aspirate the final wash thoroughly and place the cells on ice. Quickly add 0.5 ml of ice-cold 4% perchloric acid and mix gently to lyse the cells. Incubate 5 min on ice.

2a. Scrape the cells into the acid solution using a cell scraper and quantitatively transfer the lysate to a screw-cap microcentrifuge tube. Microcentrifuge 5 min at maximum speed, 4°C, to bring down the cell debris.

3a. Transfer the supernatant to a fresh tube and keep on ice. Freshly prepare a mixture of 1.2 parts tri-*n*-octylamine to 1 part Freon (on a v/v basis) and mix well. Add 0.6 ml of this mixture to the perchloric acid supernatants, cap the tubes tightly, and vortex for at least 1 min. Extract four to six standard ATP samples of known concentration in parallel with the test supernatants.

It is very important that the samples be mixed extremely thoroughly at this point to ensure complete neutralization.

For labeled cell lysates

1b. In a 5-ml polypropylene tube add an equal volume of ice-cold 8% perchloric to the amount of labeled cell lysate required and place on ice. Mix thoroughly and incubate 5 min on ice.

2b. Centrifuge 5 min (or until protein is completely pelleted) at 3800 × *g*, 4°C.

3b. Transfer the supernatant to a fresh tube and keep on ice. Freshly prepare a mixture of 1.2 parts tri-*n*-octylamine to 1 part Freon (on a v/v basis) and mix well. Add 1.2 vol of this mixture to the volume of sample remaining after protein precipitation and

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vortex extremely well. Extract four to six standard ATP samples of known concentration in parallel with the test supernatants.

It is very important that the samples be mixed extremely thoroughly at this point to ensure complete neutralization.

Recover neutralized aqueous sample

4. After complete mixing, centrifuge 5 min at 3800 × *g*, room temperature.

   Three layers should be evident following centrifugation. The bottom layer is excess tri-n-octylamine/Freon, the central layer is octylamine perchlorate (which is often yellow in color), and the upper layer is the neutralized aqueous sample.

5. Transfer the upper layer to a fresh tube and measure the pH using pH paper.

   The pH should be pH 6 to 7. If the pH of the sample is <6, repeat the treatment with tri-n-octylamine/Freon, although this is rarely necessary.

Quantitate ATP concentration

6. Equilibrate the SAX column in water for 30 min at 1 ml/min flow rate.

   As an alternative to this step and subsequent steps it is possible to quantitate ATP using a luminescent assay. A very easy, sensitive and convenient assay for ATP is available from Sigma and is based on using the enzyme luciferase, which produces light from luciferin and ATP. Hence, at constant luciferin/luciferase concentrations, standard curves can be constructed for the quantum light yield at different ATP concentrations. Unknown samples derived from cell lysates can thus be easily quantitated. If, however, one needs to determine the specific radioactivity of the γ phosphate of ATP (as for $^{32}P_i$-labeled cells), proceed as described in this protocol .

7. Subject the column to a blank gradient run—i.e., run the linear gradient to be used (from 100% solution A to 100% solution B) through the column but do not inject the sample.

   A typical simple gradient is shown in Table 13.8.1. The initial 5 min allows the contents of the injection loop to be cleared and passed onto the column, assuming use of up to a 5-ml-volume loop. It is very important that the ionic strength of the sample be <200 mM for the inorganic phosphate to bind to the column. In practice, sample loops of up to 5-ml volume can be used successfully, allowing 0.5-ml samples to be diluted 10-fold with water before analysis.

Table 13.8.1 Gradient Profile for the Separation of Nucleotides by HPLC and Approximate Retention Times for Selected Nucleotides[a,b]

Time (min)	%A	%B	Approximate retention time (min)		
			AMP/GMP	ADP/GDP	ATP/GTP
0	100	0			
5	100	0			
70	40	60	30/35	45/52	75/85
85	40	60			
90	0	100			
120	0	100			
125	0	0			

[a] A = Milli-Q water; B = 1.25 M $NH_4H_2PO_4$ buffer, pH 3.8.

[b] Column = Whatman Partisphere SAX, 25 cm × 4.6–mm i.d. The retention times for the nucleotides are approximate, as different HPLC systems produce slight differences in gradient delivery and different HPLC columns give different separations.

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8. Filter standard mixture of nucleotides through a low-dead-volume syringe filter, then run on the SAX column.

   A good mix to use is AMP, ADP, ATP, GMP, GDP, and GTP, all at a concentration of 1 mg/ml. A 10-ml injection will result in easily detected peaks at 254 nm using the UV detector. Once consistent separation of the required standard nucleotides is made, the samples can be analyzed.

9. Filter the four to six ATP samples of known concentration (from step 5) through a low-dead-volume syringe filter and inject them into the SAX column. Construct a standard curve.

   The unknown samples should fall around the center of the curve. In practice, this can be estimated according to the fact that the intracellular concentration of ATP is normally ~1 mM, so the starting cell volume can be used to roughly estimate the ATP concentration in the cell lysates.

10. Filter the unknown samples (from step 5) through a low-dead-volume syringe filter, then run them on the SAX column and collect the ATP peak (which absorbs at 254 nm) using a fraction collector connected in series after the UV monitor.

    The concentration of ATP is then calculated.

11. Transfer an aliquot of the ATP peak to a scintillation vial and determine cpm.

    The amount of [γ-^{32}P]ATP is then calculated from the amount of ^{32}P associated with the peak absorbing at 254 nm.

SUPPORT PROTOCOL 2

DETERMINATION OF THE SPECIFIC ACTIVITY OF THE γ-PHOSPHATE OF [^{32}P]ATP

The above technique (see Support Protocol 1) details methods for the determination of the total ATP concentration and the amount of ^{32}P associated with that concentration. For the case of permeabilized cells where [γ-^{32}P]ATP has been introduced specifically as the phosphate donor, the amount of radioactivity associated with ATP will give the specific activity of the γ phosphate. However, when cells are labeled with [^{32}P]inorganic phosphate ($^{32}P_i$) as in *UNIT 13.2*, the ^{32}P will be distributed between all of the phosphorus atoms in ATP and hence the total radioactivity will not give a meaningful result regarding the specific activity of the γ phosphate of ATP. Also, in permeabilization studies such as the ones described in this unit, it is good to know the final specific activity of the ATP because the [γ-^{32}P]ATP is diluted with the ATP contained within the cells. A good method for obtaining this specific activity information is the one developed by Hawkins et al. (1983), which uses cyclic AMP–dependent protein kinase (cA-PrK) to phosphorylate the substrate histone 2A under conditions where the purified [^{32}P]ATP is the sole ATP in the assay. The ^{32}P-labeled ATP is completely used up during the phosphorylation of histone. Phosphorylated histone is easily separated from other ^{32}P-containing metabolites; hence the amount of ^{32}P associated with the γ phosphate of the labeled ATP is easily determined. The method detailed below is a slight modification of the Hawkins method and uses a peptide substrate in place of histone to allow greater specificity. This procedure is the basis for many protein kinase assays and is described in greater detail in *UNIT 13.7*. The details of assays for cA-PrK are dealt with in detail in that unit, along with specifics about assay buffers and substrate preparation.

Materials

Cell lysates containing unknown [^{32}P]ATP concentrations
cA-PrK (purified subunit from bovine heart; Calbiochem) and 10× cA-PrK assay buffer (see recipe)
0.5 mM cyclic AMP (cAMP; Calbiochem)

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10 mg/ml kemptide (Sigma)
1 M DTT (store at –20°C)
0.5 M $MgCl_2$
75 mM orthophosphoric acid
Acetone

Screw-cap microcentrifuge tubes
P 81 phosphocellulose paper, precut and labeled (*UNIT 13.7*, Support Protocol 3)
30°C circulating water bath

Additional reagents and equipment for precipitating ^{32}P-labeled peptide on P 81 paper (*UNIT 13.7*, Support Protocol 3)

1. For each lysate to be assayed, prepare the following reaction mixture in a screw-cap microcentrifuge tube on ice:

   25 µl [γ-^{32}P]ATP-containing lysate
   5 µl 10× cA-Prk assay buffer
   1 µl 0.5 mM cAMP
   5 µl 10 mg/ml kemptide
   1 µl 1 M DTT
   1 µl 0.5 M $MgCl_2$
   2 µl H_2O.

   Once all assay tubes have been set up and labeled, cap tubes and keep on ice.

2. Start one reaction every 30 sec by directly adding 15 U cA-PrK to the reaction mixture and transferring the tube to a 30°C water bath. Incubate for the optimal period of time.

   Because the objective is to transfer all of the γ-phosphate of ATP if possible, a relatively large amount of cA-PrK is required. Initially, 15 U per tube can be used; this can be adjusted for later experiments.

   The optimal assay time must be determined empirically by performing a time course. Times of 60, 120, 180, and 240 min should be tried; the time chosen should be one after the incorporation of ^{32}P into kemptide has reached a plateau.

3. Pipet 30 µl of the contents of each tube onto the surface of prepared P 81 paper squares (see *UNIT 13.7*, Support Protocol 3). Immediately place the squares in a beaker of 75 mM orthophosphoric acid.

4. Once all the assay tubes have been treated as in step 3, wash the P 81 paper squares five times with 75 mM orthophosphoric acid (see *UNIT 13.7*, Support Protocol 3).

5. Fill the beaker with acetone and wash the papers an additional 5 min (see *UNIT 13.7*, Support Protocol 3). Discard the acetone and allow the papers to dry at room temperature.

6. Place each square of P 81 paper in a separate 20-ml scintillation vial and determine the associated radioactivity using a scintillation counter.

   Once the number of dpm of ^{32}P transferred is known, it is a simple task to calculate the specific activity of the ATP input into the experiment. For example—if the amount of total ATP has been determined to be 10 µmol, the radioactivity associated with ATP is 220,000 dpm, the amount of ^{32}P transferred to kemptide is 110,000 dpm, and the amount of ^{32}P remaining is 110,000 dpm—then there are 125,000 dpm of ^{32}P in the γ-phosphate of ATP as there are ~2.2 × 10^6 dpm per µCi. Hence, 110,000 dpm is equivalent to 0.05 µCi, so the specific activity of the γ-phosphate of ATP is 0.5 mCi/mmol.

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REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

cA-PrK assay buffer, 10×

500 mM Tris·Cl, pH 7.5 at 30°C (*APPENDIX 2E*)
50 mM $MgCl_2$
Store up to 6 months at –20°C

Cold ATP stock solution, 10 mM

Mix equal volumes of 20 mM $MgCl_2$ and 20 mM disodium ATP. Slowly raise the pH of the solution to 7.4 with 1 M NaOH using constant stirring. Filter sterilize and store in small aliquots up to 1 year at –20°C.

Extracellular buffer

110 mM NaCl
10 mM KCl
1 mM $MgCl_2$
1.5 mM $CaCl_2$
30 mM HEPES
10 mM glucose
Filter sterilize and store up to 1 month at 4°C

Intracellular buffer

25 mM $NaHCO_3$
120 mM KCl
1 mM KH_2PO_4
10 mM $MgCl_2$
20 mM HEPES
10 mM glucose
Filter sterilize and store up to 1 month at 4°C

Lysis buffer for immunoprecipitation, 2×

100 mM HEPES
200 mM NaCl
40 mM EDTA
4 mM EGTA
100 mM NaF
20 mM β-glycerophosphate
2 mM Na_3VO_4
2% (v/v) NP-40 or Triton X-100
Filter sterilize and store up to 6 months at 4°C

This buffer has a 10× concentration of EDTA to chelate the excess magnesium used in kinase reactions.

PBS/BSA

Dissolve 10 mg bovine serum albumin (BSA) in 100 ml PBS (*APPENDIX 2E*). Filter sterilize using a low-protein-binding membrane (e.g., 0.2-µm Millipore GV filters) and store up to several months at 4°C.

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Permeabilization buffer

120 mM KCl
25 mM $NaHCO_3$
5 mM HEPES
10 mM $MgCl_2$
1 mM KH_2PO_4
1 mM EGTA
300 mM $CaCl_2$
Filter sterilize and store up to several months at 4°C

This buffer has a free ionized calcium concentration of ~100 nM.

SDS-PAGE sample buffer, 2×

4 ml 0.125 M Tris·Cl, pH 6.8 (*APPENDIX 2E*)
4 ml 10% (w/v) SDS
1 ml glycerol
0.4 ml 2-mercaptoethanol
0.6 ml H_2O
20 mg bromphenol blue
Filter sterilize and store up to 6 months at –20°C

The pH of the Tris buffer is measured at room temperature.

Streptolysin O solution

600 U/ml stock solution: Make up a stock solution of 600 U/ml streptolysin O in PBS (*APPENDIX 2E*) and snap-freeze in liquid nitrogen. Store at –70°C in small aliquots up to several months.

60 U/ml working solution: Immediately before the experiment, dilute the 600 U/ml stock solution to 60 U/ml with PBS/BSA (see recipe). Keep on ice until ready to use.

Two-dimensional-PAGE lysis buffer

20 mM Tris·Cl, pH 8.0 (*APPENDIX 2E*)
0.3% (w/v) SDS
1% (v/v) 2-mercaptoethanol
Filter sterilize and store up to 6 months at –20°C

Two-dimensional-PAGE sample buffer

6 g urea
4 ml NP-40
0.5 ml 40% ampholytes of the required pH range (*UNIT 10.2*)
0.154 g DTT
1.12 ml H_20
Filter sterilize and store in small aliquots up to 1 month at –70°C

NOTE: *Use the highest-purity urea available so that the concentrations of the breakdown products of urea—carbamic acid and ammonia—are minimized. This is important because proteins can be carbamylated in solution by these breakdown products, thereby altering the native protein's net charge and hence its isoelectric point.*

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COMMENTARY

Background Information

The permeabilization of cells has become a popular method for the study of the regulation of signal-transduction mechanisms. In practice, similar methodologies can be used to study the different types of receptor-regulated intracellular communication mechanisms such as those regulated by heterotrimeric G-protein–coupled receptors, receptor tyrosine kinases, and cytokine receptors (Hawkins et al., 1983; Stephens and Downes, 1990; Alexander et al., 1992; Stutchfield and Cockcroft, 1993; Slowiejko et al., 1994; Stephens et al., 1994; Cunningham et al., 1995; Martys et al., 1995; Taylor et al., 1995). Although permeabilization protocols are very simple in theory, they are very much more problematic in practice. This is because different cells have different permeabilization properties; hence each time permeabilization protocols are applied to a new cell type, it is neccesary to reexamine the experimental protocol in detail before reproducible data may be obtained. Similarly, different permeabilization-inducing agents are available, but they are not easily interchangeable, nor do they show predictable properties in the maintainence of signal-transduction processes in different cell types. The current agent of choice is streptolysin O, a bacterial toxin from the organism *Streptococcus pyogenes*. Streptolysin O has been used to study G-protein–coupled receptors and receptor tyrosine kinases; for these systems it appears to work quite reproducibly (Hawkins et al., 1983; Stephens and Downes, 1990; Alexander et al., 1992; Stutchfield and Cockcroft, 1993; Slowiejko et al., 1994; Stephens et al., 1994; Cunningham et al., 1995; Martys et al., 1995; Taylor et al., 1995).

A series of experiments utilizing cell permeabilization will not produce quick results. It will, however, produce data not normally obtainable using other experimental approaches.

A number of preliminary experiments must be performed to determine the optimal conditions for the permeabilization of intact cells, as well as for the time of addition for various reagents. Aspects to be considered include the following.

1. The kinetics of phosphorylation of the protein under study in intact cells must be determined.

2. The time required for cold ATP/[γ-^{32}P]ATP equilibration with intracellular processes—e.g., phosphatidic acid formation and phosphatidylinositol-4-phosphate or phosphatidylinositol-4,5-bisphosphate synthesis—must be determined. The timing of the addition of [γ-^{32}P]ATP to the permeabilization medium must be optimized—e.g., should the [γ-^{32}P-]ATP be added at the same time as the permeabilization agent, or some time later?

3. If possible, the integrity and time course of receptor activation of a known signal-transduction mechanism should be determined—e.g., determine whether MAP kinase is activated under the conditions of permeabilization indicated in the experiments performed under point 2, above, as well as the optimal time for activation and how long the response is detectable.

4. The timing for addition of receptor agonists should be tested—e.g., can a receptor agonist be added at the same time as the permeabilization agent, at the same time as the [γ-^{32}P]ATP, or some time after these reagents?

5. It should be determined whether the protein under study is phosphorylated under the same conditions as the reporter activity observed under point 3, above, in the permeabilized cells.

6. The timing of addition of inhibitors/activators or other reagents should be determined, as well as their concentration for optimal function in the permeabilized cells.

Once these issues have been resolved, experiments can easily be optimized and the phosphorylation event under study examined in detail.

Critical Parameters and Troubleshooting

A number of problems can be experienced when using permeabilization protocols. High-quality streptolysin O must be used, and each preparation of streptolysin O should be tested according to the supplier's instructions before use.

The timing for addition of all reagents to the cells must be strictly reproduced within experiments and between experiments. If large variability is noted between experiments and the various timings have been strictly observed, then the problem is almost always due to the streptolysin O preparation used. The solution should then be checked per the supplier's instructions.

Another problem that may be encountered is hydrolysis of [γ-^{32}P]ATP by endogenous ATPases. This should always be assayed in any experiment by determining the production of

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[^{32}P]inorganic phosphate during the time course of the experiment. If the ATP concentration is depleted by >10% during the course of the experiment, general ATPase inhibitors can be included in the permeabilization buffer. One such inhibitor is ouabain; however the potential exists for the ATPase inhibitor to inhibit whatever process is being studied; hence more experiments are required to determine the result empirically for each inhibitor to be used.

Anticipated Results

Once the optimal conditions for permeabilization, ATP addition, agonist addition, and inhibitor addition have been determined, it is relatively straightforward to study a novel phosphorylation event. Initially, one can detect the phosphorylation of the protein under study as a result of receptor activation. If pharmacological tools are available—e.g., inhibitors/activators of specific protein kinases—it is possible to make a provisional identification of the kinase responsible for the phosphorylation event being examined. It is also possible to determine the proximity of the phosphorylation event to receptor activation.

Time Considerations

The entire permeabilization procedure can be completed in ~2 hr. After this time, the samples generated can be prepared for analysis by immunoprecipitation or directly analyzed by SDS-PAGE. Immunoprecipitation times are dependent on the antibodies being used, but high-affinity interactions should only require 1 to 2 hr of incubation at 4°C. Harvesting the immunoprecipitates with protein A–Sepharose will take 1 hr at 4°C, and the washing procedure then takes 30 min before the samples can be prepared for SDS-PAGE.

One-dimensional SDS-PAGE gels can generally be run in ~3 hr for a regular-sized gel or 1 hr for a minigel. The gels can then be stained to the required level in 1 to 2 hr before placing on a gel dryer for 1 to 2 hr. Once the gel has been stained and dried, it can be analyzed by either standard autoradiography or using a Phosphorimager. The exposure times required are dependent on the radioactivity contained in the protein bands. For regular autoradiography using an intensifying screen, it is possible to visualize a band containing a few hundred cpm with an overnight exposure at –70°C. A Phosphor screen will give similar data with ~4 hr exposure.

For two-dimensional-PAGE analysis, the samples can be placed in the lyophilizer overnight prior to electrophoresis. The two-dimensional-PAGE procedure will take one day to complete before the gel can be dried and analyzed by autoradiography or with a Phosphorimager.

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14

POST-TRANSLATIONAL MODIFICATION: SPECIALIZED APPLICATIONS

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CHAPTER 14
Post-Translational Modification: Specialized Applications

INTRODUCTION

The amino acid sequence of a protein determines its structural and functional properties. Yet the primary structure of a polypeptide is not a complete description of its chemical constituents; modifications of the polypeptide backbone may alter the intracellular destination, as well as modify functional properties of the polypeptide in question. Some of these modifications occur as the polypeptide rolls off the ribosome; others take place on complete polypeptide chains; or both co- and post-translational modification may occur on the same polypeptide, as exemplified by protein-bound carbohydrates.

A number of post-translational modifications may be inferred from the primary structure or from the open reading frame established by DNA sequencing. These include potential cleavage sites for signal peptidase in the case of secretory or type-I membrane proteins; cleavage sites for other proteases that process inactive precursors into active polypeptides, as exemplified by neuropeptide precursors; consensus sequences for N-linked glycosylation; and C-terminal modification by isoprenoid moieties in conjunction with carboxymethylation. Similarly, consensus sequences for certain types of phosphorylation events have been established. Although for many of these modifications consensus sequences indeed indicate their possible occurrence, it is not always safe to conclude that such modifications therefore necessarily take place. For that reason, methods to identify and characterize these modifications are essential.

All of the naturally occurring amino acids can occur in proteins in a modified form. In this manual, separate chapters are devoted to the most intensively and possibly widespread protein modifications, glycosylation (Chapter 12) and phosphorylation (Chapter 13). In this chapter, methods for the study of other protein modifications will be addressed.

The presence of disulfide bonds stabilizes the structure of proteins and allows them to survive in the extracellular milieu. This type of modification cannot be inferred from primary structure without the aid of homologous proteins for which the disulfide bonding pattern has been established by chemical analysis, as described elsewhere in this manual (*UNIT 7.3*). Furthermore, in guiding protein folding and in allowing the formation of higher-order structures such as homo- or hetero-oligomers, disulfide bond formation is an important aspect of how the conformation of a mature, functional protein is reached. *UNIT 14.1* describes the technology with which to study disulfide bond formation in living cells and in a completely cell-free system.

Modification of proteins by lipid moieties allows proteins to interact with membranes. The functional properties of a number of proteins depend critically on this modification, and establishing whether or not acylation (*UNIT 14.2*) or isoprenylation (*UNIT 14.3*) have occurred is therefore important. There are other modifications that occur at distinct subcellular localizations, such as the modification of tyrosine with sulfate. Future supplements will deal with the most prevalent of these post-translational modifications, and aid the investigator in achieving a more complete description of the protein under study.

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Analysis of Disulfide Bond Formation

Most proteins synthesized in the endoplasmic reticulum (ER) in eukaryotic cells and in the periplasmic space in prokaryotes are stabilized by disulfide bonds. Disulfide bonds are of two types: intrachain (within a polypeptide chain) and interchain (between separate chains). Intrachain disulfide bonds are formed during cotranslational and post-translational folding of a newly synthesized protein. Most interchain disulfide bonds are formed at a later stage in the maturation process and establish covalent links between subunits in oligomeric proteins.

Disulfide bond formation can be followed in cultures of intact cells (Basic Protocol 1 and Alternate Protocols 1 and 2) or in an in vitro translation system containing isolated microsomes (Basic Protocol 2 and Alternate Protocol 3). First, the newly synthesized protein of interest is biosynthetically labeled with radioactive amino acids in a short pulse. The labeled protein is chased with unlabeled amino acids. At different times during the chase, a sample is collected, membranes are lysed with detergent, and the protein is isolated by immunoprecipitation (Support Protocol 1). The immunoprecipitates are analyzed for the presence of disulfide bonds by SDS-PAGE with and without prior reduction (Support Protocol 2). The difference in mobility observed between the gels with unreduced and reduced samples is due to disulfide bonds in the unreduced protein.

CAUTION: Radioactive materials require special handling. See *APPENDIX 2B* concerning safe use of radioisotopes.

ANALYSIS OF DISULFIDE BOND FORMATION IN INTACT MONOLAYER CELLS

BASIC PROTOCOL 1

In vivo, disulfide bond formation can be examined in cells growing in monolayers on tissue culture dishes (Braakman et al., 1991). This allows multiple wash steps in a short period of time and makes it easy to use short pulse and chase times. In addition, adherent cells are suitable for studies requiring frequent changes of medium with very different composition. When cells do not adhere or when low volumes of medium are desirable (e.g., when expensive additives are needed), the analysis can be done in suspension (see Alternate Protocol 1). This method detects cotranslational and post-translational disulfide bond formation; it is also possible to analyze post-translational disulfide bond formation (see Alternate Protocol 2).

Materials

Adherent cells
Tissue culture medium containing methionine, 37°C
Wash buffer (see recipe), 37°C
Depletion medium (see recipe), 37°C
Labeling medium (containing 125 to 250 μCi/ml [^{35}S]methionine; see recipe), 37°C
Chase medium (see recipe), 37°C
Stop buffer (see recipe), 0°C
Lysis buffer (see recipe), 0°C

60-mm tissue culture dishes, sterile
37°C humidified 5% CO_2 incubator
37°C water bath with rack or insert to hold tissue culture dishes (e.g., Unwire racks for 15- and 50-ml tubes, Nalge)
Aspiration flask for radioactive waste
Flat, wide ice pan with fitted metal plate (e.g., VWR Scientific)
Cell scraper

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Additional reagents and equipment for immunoprecipitation (see Support Protocol 1) and nonreducing and reducing SDS-PAGE (see Support Protocol 2)

NOTE: The volumes described here are for a 60-mm dish of cells. Volumes must be adjusted, based on the surface area of the dish, for other sizes.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Perform pulse and chase

1. Set up cultures of adherent cells in tissue culture medium containing methionine in 60-mm tissue culture dishes so the cells will form a subconfluent monolayer on the day of the experiment. Incubate at 37°C.

   The experiment requires at least 1 dish per time point, and each dish should contain ≥10^6 cells.

2. Set up 37°C water bath with rack or insert for tissue culture dishes. Check that the water level is in contact with the bottom of a tissue culture dish but does not allow it to float if the lid is removed. Arrange the aspiration flask for radioactive waste so the pipet easily reaches dishes in the water bath.

3. Rinse cells twice with 2 ml wash buffer. Aspirate wash buffer and add 2 to 2.5 ml depletion medium. Incubate 15 to 30 min at 37°C in an incubator.

   This step depletes methionine in the cultured cells.

4. Remove dishes from the incubator and place on rack in 37°C water bath.

5. Pulse-label the cells, one dish at a time: aspirate depletion medium, add 400 µl labeling medium containing 50 to 100 µCi [^{35}S]methionine, and incubate 1 to 2 min on rack in 37°C water bath.

   The pulse time should be equal to or shorter than the time required to synthesize the protein of interest (assuming a rate of 4 to 5 amino acid residues/sec) and long enough to detect the protein.

   If the protein of interest does not contain many methionines, or if it contains several cysteines, it may be worthwhile to deplete cysteine as well as methionine and label with [^{35}S]methionine + [^{35}S]cysteine (50 to 100 µC; the total ratio depends on the protein) or with the same quantity of unpurified [^{35}S]methionine, which usually contains ~15% (v/v) [^{35}S]cysteine as well. The stabilized form of unpurified methionine (e.g., [^{35}S]in vitro labeling mix, Amersham) is less volatile and should be used for short pulses in a water bath to minimize radioactive contamination of air, pipets, and equipment.

For 0-min chase interval

6a. Add 2 ml chase medium to stop the pulse at precisely the end of the labeling interval. Rock gently to mix.

7a. Aspirate chase medium as quickly as possible. Transfer dish to aluminum plate on ice pan. Immediately add 2.5 ml cold stop buffer to end the chase.

For all other chase intervals

6b. At precisely the end of the pulse labeling interval, add 2 ml chase medium to start the chase. Rock gently to mix. Aspirate medium and add 2 ml chase medium again. Incubate in a 37°C incubator or 37°C water bath for the desired chase intervals.

   Choice of chase times is determined by the time it takes a protein to fold and form disulfide bonds. Generally, the first chase interval is equal to pulse time, the next approximately double that, and so on (e.g., 2, 5, 10, 20, and 40 min).

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For short chase intervals, dishes can be incubated in a water bath, but for chase intervals >15 to 20 min, pH is best maintained in an incubator.

7b. At the end of the chase interval, aspirate chase medium and transfer dish to aluminum plate on ice pan. Add 2.5 ml cold stop buffer to end the chase.

Cold stop buffer is used to stop all cellular processes. Cells may be left ≤30 min on ice in stop buffer.

Prepare the lysate

8. Remove stop buffer and add 2.5 ml cold stop buffer.

9. Aspirate dish as dry as possible. Add 600 µl cold lysis buffer.

No incubation with lysis buffer is necessary when the buffer contains an alkylating agent. If alkylating agent is omitted from the lysis buffer, incubate 10 min with 20 mM N-ethylmaleimide (NEM) or longer with 20 mM iodoacetamide or 20 mM iodoacetic acid (typically 15 to 45 min).

10. Scrape dish and mix cell lysate with cell scraper. Transfer the lysate to a labeled 1.5-ml microcentrifuge tube.

11. Microcentrifuge 5 min at 12,000 rpm, 0°C, to pellet nuclei. Transfer supernatant (postnuclear lysate) to a clean 1.5-ml microcentrifuge tube.

12. Immediately analyze postnuclear lysate by immunoprecipitation (see Support Protocol 1 and *UNIT 13.2*) and nonreducing and reducing SDS-PAGE (see Support Protocol 2) or freeze it rapidly in liquid nitrogen and store at –80°C.

Lysate with alkylating agent can be stored 1 to 2 hr on ice; lysate without alkylating agent should not be stored >5 min on ice.

ANALYSIS OF DISULFIDE BOND FORMATION IN CELLS IN SUSPENSION

ALTERNATE PROTOCOL 1

When cells do not adhere adequately to a solid support, or when it is desirable to use small volumes of reagents for analyses, cells in suspension may be used to analyze disulfide bond formation. One advantage to this approach is that samples at different chase intervals may be collected from a single tube of labeled cells. However, wash steps cannot be included during the pulse-chase incubations because the washes take too much time. Prior to starting the experiment it is necessary to determine (1) the minimum volume for incubating the cells (x µl), (2) the number of chase time points (y), and (3) the desired sample volume (z µl). Approximately 10^6 cells should be used for each time point.

***Additional Materials** (also see Basic Protocol 1)*

Culture of suspension cells >1000
10 mCi/ml [^{35}S]methionine (>1000 Ci/mmol; Amersham)
2× lysis buffer (see recipe)
Concentrated chase medium (see recipe)

50-ml polystyrene tube with cap, sterile
Beckman GPR cell centrifuge or equivalent

Additional reagents and equipment for immunoprecipitation (see Support Protocol 1) and nonreducing SDS-PAGE (see Support Protocol 2)

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: Keep cells in suspension during incubations by gently swirling the tube at regular intervals.

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1. Transfer suspension cells for analysis to a sterile 50-ml polystyrene tube with cap.

   Use ~10^6 cells per data point.

   If it is necessary to minimize the volumes of reagents used for the experiment, adherent cells can be removed from the dish by treating them with 0.25% (w/v) trypsin/0.2% (w/v) EDTA and resuspending them in a low-calcium medium.

2. Centrifuge cells 4 min at 500 × *g* (1500 rpm), 20° to 37°C. Resuspend pellet in 2*y* ml depletion medium and centrifuge again. Resuspend in 2*y* ml depletion medium. Incubate 15 to 30 min at 37°C.

   Pelleting conditions may vary with cell type.

3. Centrifuge cells 4 min at 500 × *g*, 20° to 37°C. Resuspend cells in *x* µl depletion medium in appropriate tube and place in water bath.

4. At the start of pulse, add 50 to 100 µCi undiluted [^{35}S]methionine per time point to the cell suspension and mix by swirling. Incubate for the pulse period.

5. At the end of the pulse, add ≥4 vol (4 × *x* µl) concentrated chase medium. Mix by swirling.

   The total volume after addition of concentrated chase medium should be slightly more than y × z µl to allow for loss due to evaporation.

   The final concentrations of chase medium components must be identical to those for the chase medium used in Basic Protocol 1 (see recipe for chase medium). Calculate the concentrations needed for the concentrated chase medium. A 1.25× solution is appropriate if 4 vol concentrated chase medium is added to the labeling mixture; for other added volumes, the concentrations must be adjusted accordingly.

6. Immediately collect the first sample of volume *z*. Add an equal volume of 2× lysis buffer, mix well, and place on ice.

7. At every chase time point, take a sample, add an equal volume of 2× lysis buffer, mix well, and place on ice.

   After the sample for the last chase time point is collected, the tube of cells should be almost empty.

8. Immediately analyze samples by immunoprecipitation (see Support Protocol 1) and nonreducing and reducing SDS-PAGE (see Support Protocol 2) or freeze rapidly in liquid nitrogen and store at –80°C.

ALTERNATE PROTOCOL 2

ANALYSIS OF POST-TRANSLATIONAL DISULFIDE BOND FORMATION IN INTACT CELLS

To determine the effect of certain conditions, such as ATP depletion, on folding, it may be necessary to separate the folding process from translation. This can be done by delaying folding until translation, glycosylation, and signal-peptide cleavage are complete. Folding of a disulfide-bonded protein may be prevented by incubating cells in reducing agent. Newly synthesized proteins cannot form disulfide bonds and often cannot fold when oxidation is prevented. When the reducing agent is removed after pulse-labeling, formation of disulfide bonds and folding may proceed (Braakman et al., 1992a,b; see Fig. 14.1.1).

Wash the cells and deplete the methionine (see Basic Protocol 1, steps 2 and 3). Add dithiothreitol (DTT; *APPENDIX 3A*) to labeling medium to give a final concentration of 5 mM. Perform a pulse-chase experiment and prepare lysates (see Basic Protocol 1, steps 4 to 11).

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It may be necessary to optimize the final concentration of DTT in labeling medium for each cell type. During long incubations, DTT may affect cellular ATP levels. Efficiency of incorporation may also be slightly diminished by the presence of DTT. The duration of pulse labeling may be increased to improve incorporation of label in protein.

Before the effects of different conditions on folding are tested, the efficiency, rate, and outcome of post-translational folding should be compared to those of cotranslational folding.

ANALYSIS OF DISULFIDE BOND FORMATION IN ROUGH ENDOPLASMIC RETICULUM–DERIVED MICROSOMES

BASIC PROTOCOL 2

Formation of disulfide bonds in proteins which possess signal sequences that target the protein to the endoplasmic reticulum can also be studied with an in vitro translation/rough endoplasmic reticulum–derived microsome translocation and folding system (Fig. 14.1.1). ^{35}S-labeled proteins are generated by the translation of mRNA with a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Translation is carried out in the presence of dog pancreas microsomes; this combination permits cotranslational insertion of ^{35}S-labeled protein into microsomes. An oxidizing agent (i.e., oxidized glutathione, GSSG; *APPENDIX 3A*) is present during translation to allow monitoring of cotranslational or vectorial oxidation from the N- to the C-terminus of the protein, the mechanism by which proteins acquire their disulfide bonds under normal physiological conditions.

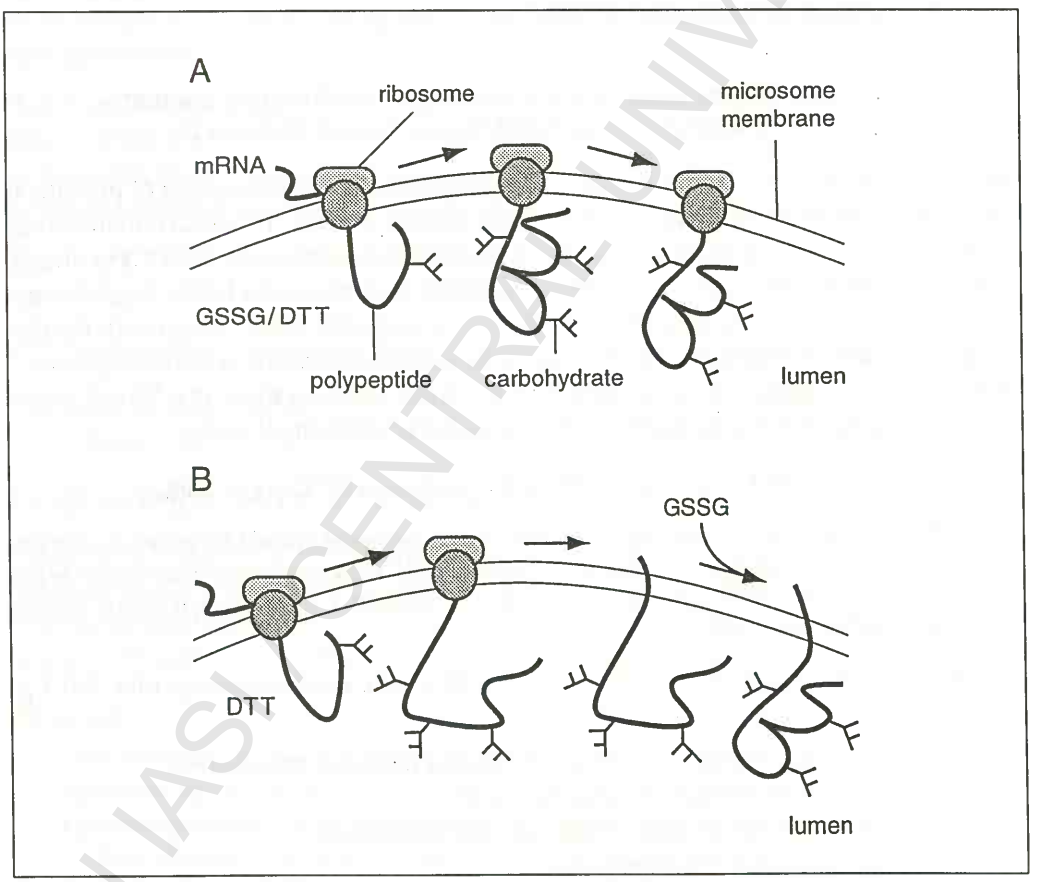

Figure 14.1.1 In vitro translation/rough endoplasmic reticulum–derived microsome translocation and folding system. (**A**) For cotranslational folding, the protein is translocated into microsomes containing an oxidizing environment (GSSG). This provides an opportunity for the protein to fold vectorially during the translation and translocation processes. (**B**) For post-translational folding, oxidizing agent is added after translation and translocation, permitting synchronization and isolation of the folding process.

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Oxidation of proteins in microsomes provides a system where the experimental conditions can be more extensively controlled and manipulated. The oxidation state of the protein can be trapped by alkylation with *N*-ethylmaleimide (NEM) and monitored by immunoprecipitation (see Support Protocol 1) and nonreducing and reducing SDS-PAGE (see Support Protocol 2).

Materials

1 equivalent/µl nuclease-treated dog pancreas microsomes (Promega)
Rabbit reticulocyte lysate treated with ATP-regenerating system and nucleases (Promega)
1 mM amino acid mixture lacking methionine (Promega)
10 mCi/ml [^{35}S]methionine (1000 Ci/mmol, Amersham)
100 mM dithiothreitol (DTT)
RNase-free H_2O (e.g., DEPC-treated; see recipe)
23 U/ml RNase inhibitor (e.g., RNasin, Promega)
1 µg/µl mRNA for the protein of interest
100 mM oxidized glutathione (GSSG; *APPENDIX 3A*), titrated to neutrality with KOH
120 mM *N*-ethylmaleimide (NEM) in 100% ethanol (prepare from 1 M stock; see recipe)
Lysis buffer (see recipe)
2× SDS sample buffer (*UNIT 10.1*; optional)

27°C water bath
1.5-ml microcentrifuge tubes, RNase-free
Ice bath

Additional reagents and equipment for immunoprecipitation (see Support Protocol 1) and nonreducing SDS-PAGE (see Support Protocol 2)

NOTE: The major source of failure in the in vitro translation/translocation of proteins is contamination with ribonucleases (RNases) that degrade mRNA. To avoid contamination, water and salt solutions should be treated with diethylpyrocarbonate (DEPC) to chemically inactivate RNases; glass and plasticware should be treated with DEPC-treated water or otherwise treated to remove RNase activity (see recipe for DEPC treatment). Freshly opened plasticware that has not been touched by unprotected hands is also acceptable. It is also helpful to keep a set of solutions for RNA work alone to ensure that "dirty" pipets do not contaminate them. In addition, gloves should be worn at all times.

1. Thaw reagents and solutions rapidly with agitation in 27°C water bath.

   Rabbit reticulocyte lysate and dog pancreas microsomes should be stored in aliquots of working volumes (i.e., 6 µl and multiples of 6 µl). Repeated freeze-thaw cycles result in inefficient translation and translocation of protein. Freeze other reagents rapidly in liquid nitrogen after use.

2. Prepare reaction mix in an RNase-free 1.5-ml plastic microcentrifuge tube (96.7 µl total):

   6 µl 1 equivalent/µl nuclease-treated dog pancreas microsomes
   52 µl treated rabbit reticulocyte lysate
   2 µl 1 mM amino acid mixture lacking methionine
   8 µl 10 µCi/µl [^{35}S]methionine
   1 µl 100 mM DTT
   16 µl RNase-free H_2O
   4 µl 23 U/µl RNase inhibitor
   4 µl 1µg/µl mRNA for the protein of interest
   3.7 µl 100 mM GSSG.

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This reaction mix is enough for 10 samples. Adjust the volumes proportionately for more or fewer samples. Each sample represents a different time point.

mRNA for the protein of interest can be transcribed from cDNA for the protein of interest into a commercially available vector (e.g., pBluescript, Stratagene) which contains both T7 and T3 promoters for RNA polymerases.

3. Mix thoroughly with a pipet. Incubate at 27°C.

   Set pipettor to less than the total volume of reaction mixture to avoid introducing air bubbles. Mix by repeatedly taking the mixture up and gently expelling it with the pipettor.

4. At the appropriate time (e.g., 0.5, 1, 1.5, 2, 3,...*n* hr), transfer 9-µl samples to separate tubes and add 2.4 µl of 120 mM NEM in ethanol (25 mM final) to alkylate proteins. Incubate 10 min on ice.

   In vitro translation of proteins is 3- to 5-fold slower than in vivo translation, so extended time points are required.

5. Add 600 µl lysis buffer to each alkylated sample *or* add 30 µl of 2× sample buffer for direct analysis of total proteins translated.

6. Analyze lysate by immunoprecipitation (see Support Protocol 1) and nonreducing SDS-PAGE (see Support Protocol 2).

ANALYSIS OF POST-TRANSLATIONAL DISULFIDE BOND FORMATION IN ROUGH ENDOPLASMIC RETICULUM–DERIVED MICROSOMES

ALTERNATE PROTOCOL 3

Post-translational oxidation is performed by translating the protein under reducing conditions in the presence of microsomes to allow accumulation of translocated and glycosylated proteins with the signal sequence removed and lacking disulfide bonds. Oxidizing agents are added post-translationally to initiate oxidation (see Fig. 14.1.1).

Perform in vitro translation and analysis as for the previous method (see Basic Protocol 2) with the following exceptions in the indicated steps.

2. Omit oxidized glutathione (GSSG) from the reaction mixture (to make 93 µl).

3. After the initial incubation of the reaction, add 4.2 µl of 100 mM GSSG and incubate 1 hr more at 27°C before proceeding to alkylate and analyze proteins (steps 4 to 8).

IMMUNOPRECIPITATION OF LYSATES

SUPPORT PROTOCOL 1

The protein of interest is precipitated from lysates of cells or microsomes using a specific antibody bound to *Staphylococcus aureus* cells or Protein A–Sepharose beads (beads give less background but are twice as expensive as the cells). The immunoprecipitate is then analyzed by nonreducing and reducing SDS-PAGE (see Support Protocol 2).

Materials

10% (w/v) killed, fixed *Staphylococcus aureus* cells (Zymed)
Antibody against protein of interest
Lysate from pulse-chase labeled cells or microsomes (see Basic Protocol 1 or 2 or Alternate Protocol 1, 2, or 3)
Immunoprecipitation wash buffer (see recipe), 37°C
TE buffer, pH 6.8 (see recipe)
2× nonreducing sample buffer (see recipe)
200 mM dithiothreitol (DTT)
Rotator

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Additional reagents and equipment for nonreducing and reducing SDS-PAGE (see Support Protocol 2)

1. Add 60 µl of 10% killed, fixed *Staphylococcus aureus* cells to 0.5 to 100 µl antibody. Rotate 1 hr at 4°C.

   The amount of antibody used depends on the antibody and its concentration.

   The time required to bind antibody to S. aureus cells may vary from 20 min to several hours.

   Alternatively, 60 µl of 10% (w/v) Protein A–Sepharose beads, washed five times in an appropriate buffer, may be used instead of S. aureus cells.

2. Add 100 to 600 µl postnuclear lysate. Incubate 1 hr at 4°C, with shaking.

   If background is high, preincubate the lysate with S. aureus cells without antibody 1 hr at 4°C. Microcentrifuge the cells 4 min at 2000 × g, room temperature, and transfer the cleared lysate to antibody-coated S. aureus cells.

3. Pellet cells by microcentrifuging 4 min at 2000 × *g* (5000 rpm), room temperature. Remove supernatant and resuspend cells in 1 ml wash buffer. Shake 5 min. Repeat wash once and pellet cells.

   The temperature of the wash can affect the level of background; generally, the higher the temperature, the lower the background. Typical wash temperatures range from 10°C to room temperature.

   Protein A–Sepharose beads can be pelleted by centrifuging 1 min at 8000 × g (10,000 rpm), room temperature.

4. Aspirate the supernatant. Add 20 µl TE buffer. Shake 5 min or until cells are resuspended.

5. Add 20 µl of 2× nonreducing sample buffer without reducing agent and vortex. Boil 5 min at 95°C and vortex.

6. Pellet *S. aureus* cells by microcentrifuging 4 min at 12,000 × *g* (12,000 rpm) to give nonreduced sample in the supernatant.

7. Transfer 20 µl supernatant to a tube containing 2 µl of 200 mM DTT and vortex. Boil 5 min at 95°C. Microcentrifuge briefly at 12,000 × *g* to give reduced sample.

8. Analyze nonreduced and reduced samples by SDS-PAGE (see Support Protocol 2).

   Samples can be rapidly frozen in liquid nitrogen and stored at –80°C, but storage may increase background.

SUPPORT PROTOCOL 2

NONREDUCING AND REDUCING SDS-PAGE

Immunoprecipitates (see Support Protocol 1) are analyzed by nonreducing SDS-PAGE to detect changes in mobility due to disulfide bond formation.

Materials

Samples in 1× sample buffer (see Support Protocol 1)
2× nonreducing sample buffer (see recipe)
PBS (*APPENDIX 2E*)/30% (v/v) methanol
1.5 M salicylate/30% (v/v) methanol

pH paper
Whatman 3MM filter paper

Additional reagents and equipment for SDS-PAGE minigel with Laemmli buffers (*UNIT 10.1*) and Coomassie blue staining and destaining (*UNIT 10.5*)

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1. Prepare a 1- or 0.75-mm thick polyacrylamide separating and stacking minigel.

   Percent acrylamide depends on molecular weight of the protein.

2. Load 8 µl of each sample. Load 1× nonreducing sample buffer in the two lanes next to the samples.

   When nonreduced and reduced samples are to be loaded on the same gel, leave two empty lanes between the two sample types, and load empty lanes with 1× nonreducing sample buffer. If nonreduced and reduced samples need to be loaded next to each other, cool the samples and add NEM to a final concentration of 100 mM to both sets of samples. Mix and pellet the cells before loading the samples on the gel.

3. Run gel ~1 hr at 20 to 25 mA until the dye front is close to or at the bottom of the gel.

4. Stain the gel, including the stacking gel, with Coomassie blue stain and destain (see *UNIT 10.5*).

   Stacking gels often contain unreduced or aggregated protein.

5. Neutralize gel by incubating it in three changes of PBS/30% methanol, 5 min each, until pH >6. Check the pH with pH paper.

6. Treat gel 30 min in 1.5 M salicylate to enhance.

   Salicylate is used as a safer, less expensive, and faster way to enhance the signal 3- to 5-fold. Fluorography can also be enhanced using commercial solutions (e.g., Enhance, DuPont or Amplify, Amersham).

7. Dry gel onto Whatman 3MM filter paper. Autoradiograph/fluorograph at –80°C.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Chase medium

Complete tissue culture medium appropriate for the cells containing:
20 mM HEPES (sodium salt), pH 7.3 (see recipe)
5 mM methionine (see recipe)
1 mM cycloheximide (optional; see recipe)
Prepare fresh daily

Add HEPES, methionine, and optional cycloheximide from concentrated stocks (see recipes).

When labeling is done with cysteine, the chase medium should also contain 5 mM cysteine (from 1 M stock; see recipe).

The chase medium may contain fetal bovine serum or another protein source, especially when chase times are long. Protein should be omitted when more defined conditions are needed. When the reducing agent dithiothreitol (DTT) is added, proteins present in the medium will partly quench the reductant.

Concentrated chase medium

Adjust the concentration of chase medium components (see recipe) so the final concentrations are 20 mM HEPES, 5 mM methionine, and 5 mM cycloheximide after the concentrated chase medium has been added to the labeling medium for cells in suspension (see Alternate Protocol 1).

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Cycloheximide, 500 mM stock

28.1 g cycloheximide
H_2O to 100 ml
Store 1- to 2-ml aliquots ≤2 years at –20°C

Thaw and warm to room temperature to completely dissolve before using. Do not freeze and thaw more than three times.

Cysteine, 1 M stock

12.12 g cysteine
H_2O to 100 ml
Store 1- to 2-ml aliquots ≤2 years at –20°C

Thaw and warm to room temperature to completely dissolve before using. Do not freeze and thaw more than three times.

Depletion medium

Methionine-free tissue culture medium containing:
0.026 M sodium bicarbonate (2.2 g/liter)
20 mM HEPES, pH 7.3 (from 1 M stock; see recipe)
Store ≤1 year at 4°C

Diethylpyrocarbonate (DEPC) treatment of solutions and labware

CAUTION: Wear gloves and use a fume hood when working with DEPC because it is a suspected carcinogen.

Solutions: Add 0.2 ml DEPC per 100 ml of the solution to be treated. Shake vigorously to dissolve DEPC. Autoclave the solution to inactivate remaining DEPC.

Any water or salt solutions used in RNA preparation should be treated with DEPC. Note that solutions containing Tris cannot be effectively treated with DEPC because Tris reacts with DEPC to inactivate it.

Labware: Rinse glass and plasticware thoroughly with DEPC solution. Alternatively, bake glassware 4 hr at 300°C; rinse plasticware with chloroform, or use fresh plasticware straight from a package that has not been touched by unprotected hands. Wear gloves for all manipulations.

Note that autoclaving alone will not fully inactivate many RNases.

Ethylenediaminetetraacetic acid (EDTA), 200 mM stock

18.61 g EDTA
180 ml H_2O
10 mM NaOH added dropwise with mixing just until EDTA dissolves
Adjust pH to 7.3
H_2O to 250 ml
Store at 4°C

For EDTA, pH 6.8, adjust the pH to 6.8.

HEPES (N-2-hydroxyethylpiperidine-N′-ethanesulfonic acid), 1 M stock

119.15 g HEPES (sodium salt)
400 ml H_2O
Adjust pH to 7.3 with 10 M NaOH
H_2O to 500 ml
Store 100-ml aliquots at –20°C and thawed aliquots at 4°C

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Immunoprecipitation wash buffer

PBS (*APPENDIX 2E*)/0.5% (v/v) Triton X-100 *or*
PBS/150 mM NaCl
Store at 4°C or room temperature

For every protein-antibody combination, the optimal wash buffer needs to be determined. The two buffers listed are particularly mild wash buffers that will maintain most antigen-antibody interactions but may lead to high background.

To decrease background, other detergents or multiple detergents may be added, salt concentration may be increased, or a combination of the two may be used. SDS at a concentration ≥0.05% (w/v) may be especially helpful, with or without the quenching effect of added nondenaturing detergents.

Labeling medium

Methionine-free tissue culture medium containing:
125 to 250 µCi [^{35}S]methionine/ml
Prepare fresh for each experiment (400 µl per chase time point)

Addition of 50 to 100 µCi in 400 µl to a 60-mm-diameter dish (~3–5 × 10^6 cells) should be sufficient to visualize a 1- to 2-min-pulse-labeled protein that is expressed to a high level in the cell.

[^{35}S]methionine + [^{35}S]cysteine or unpurified [^{35}S]methionine + [^{35}S]cysteine can be used to label proteins that contain few methionines but several cysteines.

Lysis buffer

PBS (APPENDIX 2E) or similar buffer containing:
0.5% (v/v) Triton X-100
1 mM EDTA, pH 6.8 (see recipe), added just before use
20 mM NEM (see recipe), added just before use
1 mM PMSF (see recipe), added just before use
Small peptide mixture (10 µg/ml each final; see recipe), added just before use
Use within 3 hr

Add EDTA, NEM, and protease inhibitors (PMSF and small peptide mixture) from concentrated stocks (see recipes).

For 2× lysis buffer, double the concentrations of reagents.

Depending on the protein analyzed and on the purpose of the experiment, a variety of detergents may be used. Triton X-100 may disrupt noncovalent interactions between proteins. Milder detergents that can be used include CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), deoxycholate, octylglucoside, or a mixture of detergent and lipid.

Methionine, 200 mM stock

2.984 g methionine
H_2O to 100 ml
Store 1- to 2-ml aliquots ≤2 years at –20°C

Thaw and warm to room temperature to completely dissolve before using. Do not freeze and thaw more than three times.

N-ethylmaleimide (NEM), 1 M stock

12.5 g NEM
100 ml 100% ethanol
Store 1- to 2-ml aliquots ≤2 years at –20°C protected from light

NEM is sensitive to light and to hydrolysis in water. If it is frozen and thawed too many times or exposed to too much light, the solution will turn yellow and the NEM will precipitate.

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Nonreducing sample buffer, 2×

400 mM Tris·Cl, pH 6.8
6% (w/v) SDS
20% (v/v) glycerol
2 mM EDTA, pH 6.8 (from 200 mM stock; see recipe)
0.01% (w/v) bromphenol blue

Phenylmethylsulfonyl fluoride (PMSF), 200 mM stock

3.484 g PMSF
10 ml anhydrous isopropanol
Store in 500-µl aliquots ≤2 years at –20°C

PMSF is highly unstable in water (the half life is ~30 min at 37°C and a few hours on ice). Add this stock to solution immediately before use.

Small peptide mixture stock

10 mg/ml chymostatin in dimethyl sulfoxide (DMSO)
10 mg/ml leupeptin in DMSO
10 mg/ml antipain in DMSO
10 mg/ml pepstatin in DMSO
Store in 10-µl aliquots ≤2 years at –20°C
Use at a final concentration of 10 µg/ml each

Stop buffer

Wash buffer (see recipe) containing salts only
20 mM NEM (from 1 M stock; see recipe)
Prepare fresh and store on ice <5 hr

NEM may be replaced by iodoacetamide or iodoacetic acid, which react more slowly with free –SH groups. They are less specific than NEM and penetrate cells more slowly than NEM, but they irreversibly carboxymethylate the –SH group, whereas NEM may dissociate slowly. Deblocking of –SH groups after NEM treatment is not a problem if cells are stored ≤2 hr on ice.

TE buffer, pH 6.8

10 mM Tris·Cl, pH 6.8
1 mM EDTA, pH 6.8 (from 200 mM stock; see recipe)
Store at 4°C

Wash buffer

PBS (*APPENDIX 2E*) containing 0.9 mM Ca^{2+}/0.5 mM Mg^{2+}, Earles balanced salt solution, or other isotonic buffer containing salts only.

For most cells, calcium ions are crucial to maintain adherence to the plastic dishes, especially to withstand many washes. When cost is not an issue, all washes can be performed using depletion medium. However, depletion medium may not be used as a substitute in stop buffer.

COMMENTARY

Background Information

Folding of a newly synthesized protein can be followed by assaying for conformational changes in the molecule immediately after synthesis. For this type of analysis, a large number of antibodies that recognize specific epitopes are required to analyze the changes in the complete molecule. In a mature protein that is stabilized by intrachain disulfide bonds (which is true of most proteins synthesized in the endoplasmic reticulum), the formation of these cross-links is indicative of folding (Creighton, 1986). A disulfide bond can be formed only when the conformation allows the two participating cysteines to be in close proximity. With only few antibodies available, the rate and extent of folding of a protein can be examined grossly through detection of disulfide bond

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formation. Of course, disulfide bond formation is not identical to folding; therefore, a comparison of disulfide bond formation and conformational changes is required for every protein studied.

When a disulfide bond–containing protein is prepared for SDS-PAGE, it is routinely reduced. In nonreducing denaturing polyacrylamide gel electrophoresis, however, the protein is denatured with SDS, but the disulfide bonds remain intact. The consequence is a more compact conformation of the protein, resulting in a higher electrophoretic mobility; however, in some cases the protein may bind less SDS, resulting in a lower electrophoretic mobility. Most proteins will run faster in oxidized form than in reduced form.

The choice of radioactive labeling is dictated by the desire to follow a protein from the moment of synthesis. No other method permits following the life of a protein in a cell in such detail. Any other method of detection would require bulk production of proteins, which would be very difficult and nonphysiological.

The in vitro translation system coupled with dog pancreas microsomes has been traditionally employed to study the translocation of secretory, lysosomal, and many integral membrane proteins across the membrane of the endoplasmic reticulum (Blobel and Dobberstein, 1975). However, more recently it has been shown that microsomes possess a complete set of foldases and chaperones and thus provide an environment in which proteins can be oxidized rapidly and efficiently (Scheele and Jacoby, 1982; Marquardt et al., 1993; Hebert et al., 1995). Microsomes provide a system that can be controlled and manipulated to a much higher degree than intact cells. Direct access is provided to the lysate (equivalent to the cytosol in the cell), and intralumenal components can be manipulated with ionophores, detergents, toxins, or alkaline pH (Bulleid and Freedman, 1988; Nicchitta and Blobel, 1993; Hebert et al., 1995). Post-translational oxidation of proteins (see Alternate Protocol 2 and Alternate Protocol 3) can be used in both intact cells and microsomes to isolate the oxidation process from other biosynthetic events. Together, analysis of cellular and microsomal folding can be employed to dissect the folding pathway of a protein.

Critical Parameters

Wash buffer for adherent cells should contain Ca^{2+} and Mg^{2+} to maintain adherence to the plate, especially when there are several washes. If cost is not an issue, cells can be washed in depletion medium.

Labeling medium should contain 50 to 100 μCi ^{35}S-labeled methionine for ~3–5 × 10^6 cells. If the protein of interest has few methionines, or if it contains several cysteines, a combination of [^{35}S]methionine and [^{35}S]cysteine, or unpurified labeled methionine and cysteine, may be used for labeling. When short pulses are performed in a water bath, the stabilized version of unpurified methionine (^{35}S in vitro labeling mix, Amersham) should be used to minimize contamination of air, pipets, and incubators.

Cycloheximide in the chase medium will stop elongation of unfinished nascent peptide chains. When the kinetics of disulfide bond formation are being studied, cycloheximide should be added to the chase medium; however, it should be omitted when a maximum amount of incorporated label is required (Braakman et al., 1991).

An alkylating agent—e.g., *N*-ethylmaleimide, iodoacetamide, or iodoacetic acid—must be included in the stop buffer and lysis buffer to prevent artifactual formation of disulfide bonds. Initially, it is recommended to test at least two alkylating agents and compare the results with those obtained in the absence of alkylating agent. Ideally, there should be no difference in results obtained with the various alkylating agents, except possibly a change in electrophoretic mobility due to alkylation.

SDS-PAGE should be performed under nonreducing conditions. Reducing agents should be completely absent. If a reducing environment does exist in (one of) the samples, all samples should be quenched with at least a two-fold concentration of *N*-ethylmaleimide or another alkylating agent.

The translocation process is dependent upon free sulfhydryl groups, but oxidation requires an oxidizing environment. Thus, it would seem impossible for cotranslational oxidation to occur in the in vitro translation system with microsomes. However, it is possible to simultaneously create a reducing extralumenal compartment and an oxidizing intralumenal compartment, probably due to the presence of a putative oxidized glutathione (GSSH) transporter in microsomal membranes. The redox range over which these two processes occur efficiently is small. In addition, the redox potential is directly related to the pH, so pH must be carefully controlled.

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Troubleshooting

The presence of disulfide bonds in a protein does not guarantee an electrophoretic mobility difference between reduced and oxidized forms, especially when a protein is large and disulfide bonds form small peptide loops. The largest shift can be expected at a late chase time when all disulfide bonds have been formed. If the presence of disulfide bonds is not certain and needs to be tested, the radioactive cell lysate can be treated with GSSG, followed by immunoprecipitation and analysis by nonreducing and reducing SDS-PAGE as described (see Support Protocol 1 and Support Protocol 2). Alternatively, free sulfhydryl groups can be labeled with [^{14}C]iodoacetamide before and after reduction.

When the mobility difference between reduced and oxidized protein is minimal, resolution might be improved by using a lower percentage acrylamide gel and/or changing the alkylating agent.

The treated reticulocyte lysate used here is optimized to efficiently translate a large range of mRNAs with an ATP-generating system, mixture of tRNAs, and salts. The concentration of salts greatly affects the translation efficiency. If the efficiency of translation is low, an untreated lysate should be used and optimized specifically for the mRNA for the protein of interest.

If there is difficulty in differentiating between translocated and untranslocated proteins in microsomes, untranslocated protein can be removed by protease digestion or centrifugation. Translocation of protein across microsomal membrane protects the protein from digestion by added proteases. Furthermore, dense rough microsomes can be pelleted easily by centrifugation, thereby isolating translocated proteins from extralumenal untranslocated proteins.

If the background is high in immunoprecipitation, try different immunoprecipitation wash buffers, using other detergents or multiple detergents, increased salt concentration, or a combination of both. SDS at concentrations ≥0.05% (w/v) may be especially helpful, with or without the quenching effect of added nondenaturing detergents.

Anticipated Results

In most cases, formation of disulfide bonds creates a more compact structure that results in an increase in the mobility of a protein in nonreducing SDS-PAGE (for representative results, see Braakman et al., 1992 a,b). The number of disulfide bonds and the distance between the cysteines in a sulfhydryl pair influences the observed change in mobility. During the chase, the protein will move from the more reduced position in the gel to the more oxidized position. For some proteins distinct oxidative intermediates may be observed, but for others, a fuzzy band may precede formation of native protein.

In reducing SDS-PAGE, ideally one band will be found in intact cells. Two bands will be found in the microsomal system—one for untranslocated protein and one for translocated protein. If the protein possesses a signal sequence (1.5 to 3.0 kDa) that is cleaved during translocation, a small mobility shift dependent upon the protein's molecular weight may be observed. However, differentiation of translocated and untranslocated proteins in reducing SDS-PAGE is best seen for multiglycosylated proteins, because each glycosylation step adds 2.5 kDa to the translocated protein. Modifications of oligosaccharides and other post-translational modifications can change the electrophoretic mobility as well, but these changes can be distinguished from disulfide bond formation by comparing reducing with nonreducing gels.

Time Considerations

Design and preparation of the experiment should take the most time. The very first time, this may take 1 or more days, depending on experience and available equipment. Preparation for routine experiments is around 1 hr. The pulse-chase portion requires 1 hr plus the maximum chase time. For rapidly folding proteins, folding can be over in 15 min; for others it may take hours. Cultures of cells should be started 1 or 2 days before the experiment so they will be subconfluent on the day of the experiment.

Pulse-chase, immunoprecipitation, and SDS-PAGE are optimally done in 1 day. It is possible to rapid-freeze lysates or immunoprecipitates in liquid nitrogen and store them at −80°C until further use, but freezing may lead to higher background and less reproducibility.

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Key References

Braakman et al., 1991. See above.

Describes the protocol in intact cells, results with influenza hemagglutinin, and considerations for ultra-short pulse times.

Hebert et al., 1995. See above.

Marquardt et al., 1993. See above.

Describes the protocol in microsomes.

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Analysis of Protein Acylation

Protein acylation is the covalent attachment of fatty acids to a protein; the most commonly added fatty acids are myristate (14:0) and palmitate (16:0). First, radiolabeled fatty acids are used to label eukaryotic cells in vitro (see Basic Protocol 1). The radiolabeled material produced can then be analyzed by various methods: the type of fatty acid linkage can be determined (see Basic Protocol 2), the nature of the protein-bound label can be determined to check for interconversion (see Basic Protocol 3), and the protein-bound fatty acid can be identified (see Basic Protocol 4).

BIOSYNTHETIC LABELING WITH FATTY ACIDS

BASIC PROTOCOL 1

To identify proteins that are modified with fatty acid groups, cultured cells are incubated first in medium containing sodium pyruvate, which acts as a source of acetyl-CoA and minimizes interconversion of the fatty acid to other metabolites, and then with [^{3}H]fatty acids. Fatty acids tritiated at positions 9 and 10 provide the best combination of high specific activity and detectability for in vitro labeling, and because the tritium label is far removed from the carboxyl end where β-oxidation occurs, reincorporation of label is minimized.

Materials

Cells for culture
Complete tissue culture medium appropriate for cells
Labeling medium: complete tissue culture medium containing the relevant dialyzed serum and 5 mM sodium pyruvate, 37°C
5 to 10 μCi/μl [9,10(*n*)-^{3}H]fatty acid, e.g., [9,10(*n*)-^{3}H]palmitic acid or [9,10(*n*)-^{3}H]myristic acid (30 to 60 Ci/mmol; Amersham International, American Radiolabeled Chemicals, or NEN Research Products) in ethanol
PBS, pH 7.2 (*APPENDIX 2E*), ice-cold
1% (w/v) SDS or SDS sample buffer (for SDS-PAGE, when using adherent or nonadherent cells respectively; *UNIT 10.1*) *or* RIPA lysis buffer (for immunoprecipitation; *UNIT 13.2*)
5× SDS sample buffer (see recipe)

Cell scrapers
Nitrogen gas

Additional reagents and equipment for immunoprecipitation (*UNIT 13.2*), SDS-PAGE (*UNIT 10.1*), treating a gel with sodium salicylate (*UNIT 14.3*) or DMSO/PPO solution (*UNIT 10.2*), and fluorography (*UNIT 10.2*)

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

1. On the day before the labeling experiment, split the cells into fresh complete tissue culture medium.

   Set up the cells at two split ratios; then choose the culture closest to 70% to 80% confluency for labeling.

2. The next day, replace the medium with a minimum volume of 37°C labeling medium. Incubate 1 hr.

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Cells in suspension should be used at a cell density of 10^6 to 10^7 cells/ml. For adherent cells that are 70% to 80% confluent, the minimum amount of medium necessary to cover the dish—e.g., 1.5 ml for 60-mm dishes and 3 ml for 100-mm dishes—should be used.

3. Add 5 to 10 µCi/µl [9,10(*n*)-^{3}H]fatty acid to a concentration of 50 to 500 µCi/ml. Incubate up to 24 hr.

   Cells vary in the rate and extent of incorporation (see Critical Parameters), so both the amount of label and the duration of incubation need to be optimized. Labeling cells overnight in the presence of 200 µCi/ml [^{3}H]fatty acid will maximize the chances of detecting labeled proteins. The amount of label and/or time of incubation can then be reduced if good incorporation of label is achieved, or increased if poor incorporation is attained.

   Short labeling times (e.g., pulses on the order of minutes up to 2 hr) require amounts of label at the higher end of the indicated range. In this case, uptake is relatively low and the medium plus label can be reused one or more times. The level of label in the medium can be monitored by scintillation counting. For longer incubations the interconversion of fatty acids becomes a greater problem, and the protein-bound fatty acid label should be analyzed (see Basic Protocols 3 and 4).

   If the [^{3}H]fatty acid is not supplied in ethanol or if the concentration is too low, remove the solvent by blowing nitrogen over the solution in its original container until dry and redissolve the label in ethanol at a concentration of 5 to 10 µCi/µl. Do not transfer into another container or evaporate the solvent in a plastic container, as this will cause a significant loss of label that will adhere to the side of the container.

For adherent cells

4a. Place the dish on ice and aspirate the medium. Wash the cells twice with ice-cold PBS and lyse the cells by adding 1% SDS for SDS-PAGE (*UNIT 10.1*) *or* RIPA lysis buffer for immunoprecipitation (*UNIT 13.2*), using 100 µl of 1% SDS for a 60-mm dish or 300 µl for a 100-mm dish, or 1 ml RIPA lysis buffer.

   CAUTION*: Radioactive medium and washes must be disposed of appropriately.*

5a. Using a cell scraper, remove the lysed cells from the dish and transfer them to a 1.5-ml microcentrifuge tube. Add 20 µl lysate to 5 µl of 5× SDS-PAGE sample buffer. Use all of RIPA lysate for immunoprecipitation. Resuspend immunoprecipitate in 20 µl SDS sample buffer.

   For SDS-PAGE, use DTT at a concentration ≤20 mM, and do not boil the samples, but incubate them only 3 min at 80°C; this is necessary because the thioester linkage of the fatty acid is susceptible to cleavage.

For nonadherent cells

4b. Microcentrifuge the cell suspension 1 min at 6000 rpm, 4°C, to pellet the cells. Decant the supernatant and wash the cell pellet once by resuspending it in 1 ml ice-cold PBS and centrifuging again.

5b. Lyse the cells by resuspending the cell pellet in 100 µl SDS-PAGE sample buffer for discontinuous SDS-PAGE (*UNIT 10.1*) *or* 1 ml RIPA lysis buffer for immunoprecipitation (*UNIT 13.2*) for 10^6 to 10^7 cells. Resuspend immunoprecipitate in 20 µl SDS sample buffer.

   CAUTION*: Radioactive medium and washes must be disposed of appropriately.*

   For analysis of total protein-bound fatty acid label, lyse the cells in 100 µl 1% SDS.

   For SDS-PAGE, use DTT at a concentration ≤20 mM, and do not boil the samples, but incubate them only 3 min at 80°C; this is necessary because the thioester linkage of the fatty acid is susceptible to cleavage.

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6. Analyze whole-cell lysate or immunoprecipitate on an SDS-PAGE minigel, using 20 µl lysate per lane. Store remaining lysate at –20°C.

7. Treat the gel with sodium salicylate (*UNIT 14.3*) or DMSO/PPO solution (*UNIT 10.2*). Using preflashed film, fluorograph the gel (*UNIT 10.2*) at –70°C.

   Typical exposure times are overnight to 1 month.

ANALYSIS OF FATTY ACID LINKAGE TO PROTEIN

BASIC PROTOCOL 2

To determine the type of linkage by which the [^{3}H]fatty acid is attached to the protein (i.e., thioester, oxyester, or amide linkage), the fatty acid is selectively cleaved from the protein. The most convenient method is to run replicate lanes on an SDS-PAGE gel, cut the lanes apart, and analyze each lane separately.

Materials

Lysate or immunoprecipitate from [^{3}H]fatty acid–labeled cells (see Basic Protocol 1, step 6)
0.2 M potassium hydroxide (KOH) in methanol
Methanol
1 M hydroxylamine·HCl, titrated to pH 7.5 with NaOH
1 M Tris·Cl, pH 7.5 (*APPENDIX 2E*)

Additional reagents and equipment for SDS-PAGE (*UNIT 10.1*), treating a gel with sodium salicylate (*UNIT 14.3*) or DMSO/PPO solution (*UNIT 10.2*), and fluorography (*UNIT 10.2*)

1. Run an SDS-PAGE gel (*UNIT 10.1*) using 20 µl lysate or immunoprecipitate from [^{3}H]fatty acid–labeled cells in each of four lanes.

2. Cut the four lanes apart and transfer each lane to a 15-ml tube containing one of the following solutions:

   0.2 M KOH in methanol
   Methanol
   1 M hydroxylamine·HCl
   1 M Tris·Cl, pH 7.5.

   Incubate 1 hr at room temperature with shaking.

   The 0.2 M KOH in methanol will cleave thio- and oxyesters, but not amides; 1 M hydroxylamine·HCl will rapidly cleave thioesters but will cleave oxyesters only poorly, and will not cleave amides. Methanol and 1 M Tris·Cl serve as controls.

3. Wash each gel strip three times, 5 min each time, with water. Treat the strips with sodium salicylate (*UNIT 14.3*) or DMSO/PPO solution (*UNIT 10.2*), and fluorograph using preflashed film at –70°C.

   Typical exposure times are overnight to 1 month.

   Cleavage is measured as a reduction in the fluorographic signal compared to those for controls, and can be quantitated by densitometric scanning of the lane or scintillation counting of excised bands. Bands with fatty acids linked to the protein by thioesters will be missing or greatly reduced in lanes treated with 0.2 M KOH in methanol and 1 M hydroxylamine·HCl; oxyesters will be greatly reduced or missing in the lane treated with 0.2 M KOH in methanol and may be slightly reduced in the lane treated with 1 M hydoxylamine·HCl; and amide linkages will not be affected by any of these treatments, so that proteins with amide-linked fatty acids will appear in all four lanes.

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ANALYSIS OF TOTAL PROTEIN-BOUND FATTY ACID LABEL IN CELL EXTRACT

Due to problems of interconversion of fatty acids by β-oxidation and chain elongation and of reincorporation of label into other metabolic precursors, the protein-bound label derived from [^{3}H]fatty acids should be analyzed, especially for experiments with long labeling incubations. This protocol is used to determine how much of the label has been converted into other fatty acids or metabolites during the incubation; a different procedure must be used to determine whether the fatty acid on the protein of interest is different from that added during labeling (see Basic Protocol 4).

Materials

0.1 M HCl/acetone, –20°C
Lysate from [^{3}H]fatty acid–labeled cells in 1% SDS (see Basic Protocol 1, step 4a or 5b)
1% (w/v) SDS
2:1 (v/v) chloroform/methanol
Diethyl ether
6 M HCl (concentrated HCl diluted 1:1 with H_2O)
Hexane
5 to 10 μCi/μl [9,10(*n*)-^{3}H]fatty acid standards (30 to 60 Ci/mmol; Amersham International, American Radiolabeled Chemicals, or NEN Research Products) in ethanol
90:10 (v/v) acetonitrile/acetic acid
EN^{3}HANCE spray (NEN Research Products)

15-ml polypropylene centrifuge tubes
Mistral 3000i benchtop centrifuge with swing-out four-bucket rotor or equivalent
Nitrogen gas
30-ml thick-walled Teflon container with an air-tight screw top
110°C oven
Thin-layer chromatography tank
RP18 thin-layer chromatography plate (e.g., Merck)
Kodak XAR-5 film, preflashed

Precipitate protein

1. Add 5 vol of 0.1 M HCl/acetone to 100 μl lysate from [^{3}H]fatty acid–labeled cells in 1% SDS in a 15-ml polypropylene tube. Incubate ≥1 hr at –20°C.

   This will precipitate the protein.

2. Centrifuge 10 min at 1500 × *g* (1000 rpm in Mistral 3000i swing-out rotor), 4°C, to pellet the precipitate. Remove the supernatant and allow the pellet to air dry gently.

Remove free label

3. Dissolve the pellet in a minimum volume of 1% SDS and transfer to a 1.5-ml microcentrifuge tube. Add 5 vol of 0.1 M HCl/acetone. Incubate ≥1 hr at –20°C.

4. Repeat steps 2 and 3.

   These precipitation steps concentrate the protein and remove much of the SDS and free label.

5. Add 500 μl of 2:1 chloroform/methanol and vortex. Centrifuge 10 min at 1000 rpm, 4°C, and remove the supernatant. Repeat this step at least three times until no more free label is extracted into the organic solvent, as determined by scintillation counting of the supernatant.

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6. Add 100 µl diethyl ether to the pellet and vortex. Centrifuge 10 min at 1000 rpm, 4°C, and decant the supernatant. Dry the pellet by placing the microcentrifuge tube under a gentle stream of nitrogen.

7. Place the tube into a 30-ml thick-walled Teflon container with a air-tight screw top containing 1 ml of 6 M HCl. Flush the tube and container with nitrogen. Close the lid tightly and incubate in an oven 16 hr at 110°C.

This hydrolyzes the fatty acids from the protein.

Extract hydrolyzed fatty acids

8. Extract the contents of the tube twice with 0.5 ml hexane and pool the extracts. Dissolve the residue in 0.5 ml of 1% SDS. Determine the radioactivity in the hexane extracts and in the residue.

Fatty acids will be extracted into hexane, while label incorporated into sugars and amino acids will be mainly in the hexane-insoluble residue.

9. Evaporate the hexane extracts just to dryness with a gentle stream of nitrogen. Dissolve in 2 to 5 µl of 2:1 chloroform/methanol.

It is important not to overdry the sample because it may then be difficult to dissolve.

Identify fatty acids

10. Preequilibrate a thin-layer chromatography tank with 90:10 acetonitrile/acetic acid for 15 min.

11. Spot resuspended hexane extract onto an RP18 thin-layer chromatography plate. Dilute 1 µl [9,10(*n*)-^{3}H]fatty acid standards in ethanol to give 1 µCi/µl and spot 0.5 µl in parallel lanes. Develop the plate in 90:10 acetonitrile/acetic acid. Air dry the plate.

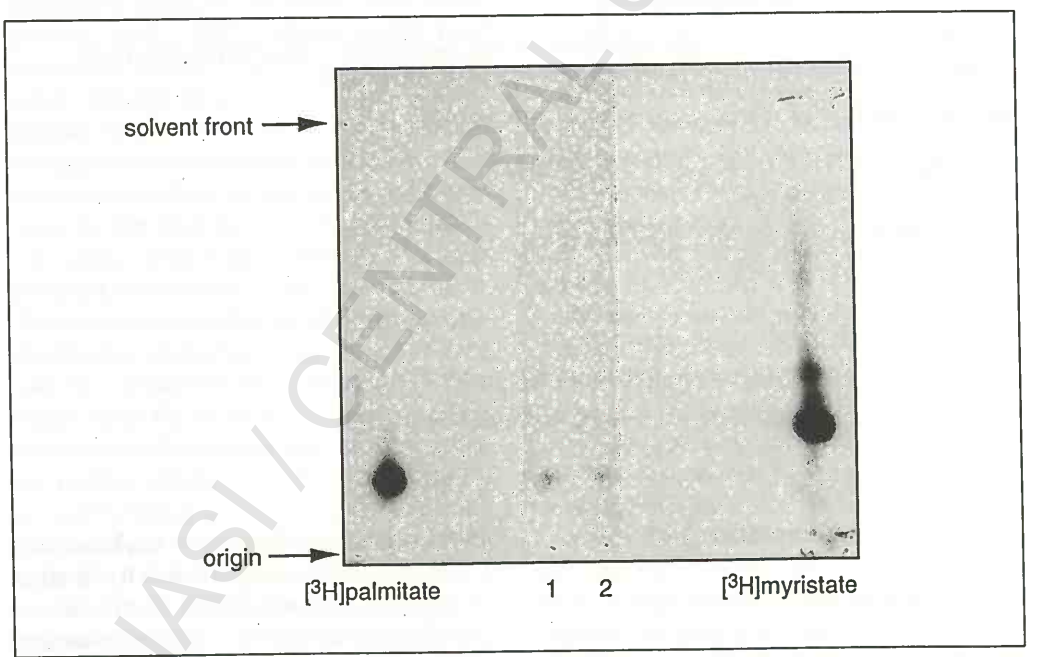

Figure 14.2.1 Fluorogram of thin-layer chromatography plate showing analysis of acylated nerve growth factor (NGF) receptor. Outside lanes, migration of 0.5 µCi [^{3}H]palmitate and [^{3}H]myristate standards. Lane 1, NGF receptor immunoprecipitated from cells labeled with [^{3}H]palmitic acid. Lane 2, NGF receptor immunoprecipitated from cells labeled with [^{3}H]myristic acid. Although the cells were labeled with different fatty acids, the protein was labeled with palmitic acid due to chain elongation of [^{3}H]myristic acid to [^{3}H]palmitic acid by the cells. Exposure for standards, 1 week; exposure for lanes 1 and 2, 1 month.

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12. Detect the radioactivity by spraying the plate with En^{3}hance spray and exposing it to preflashed Kodak XAR-5 film overnight at –70°C. Identify the fatty acids.

See Figure 14.2.1 for an example of a typical fluorogram.

BASIC PROTOCOL 4

ANALYSIS OF FATTY ACID LABEL IDENTITY

This protocol is used to identify the labeled fatty acid(s) associated with a specific protein band on an SDS-PAGE gel. Following electrophoresis, the band of interest is located either by comparison with molecular weight standards or by fluorography of a sodium salicylate–treated gel (see *UNIT 14.3*). DMSO/PPO–treated gels cannot be used. The labeled material is analyzed by thin-layer chromatography.

Materials

SDS-PAGE gel of lysate from [^{3}H]fatty acid–labeled cells
Additional reagents and equipment for analysis of protein-bound label (see Basic Protocol 3)

1. Excise the band(s) of interest from a wet or dried (fluorographed) SDS-PAGE gel. Wash three times with shaking, 5 min each, with 0.5 ml water.

   If the gel is fluorographed it should be treated with sodium salicylate (UNIT 14.3), not DMSO/PPO solution. The dried gel piece will rehydrate and the salicylate will be washed out during the washes.

2. Place the gel piece in a 1.5-ml microcentrifuge tube and lyophilize.

3. Hydrolyze the fatty acids in the band and identify them by thin-layer chromatography (see Basic Protocol 3, steps 7 to 12).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

SDS sample buffer (for discontinuous systems), 5×

3.125 ml 1 M Tris·Cl, pH 6.8 (0.313 M final)
1 g SDS (10% final)
5 mg bromphenol blue (0.05% final)
5 ml glycerol (50% final)
H_2O to 10 ml
Store at room temperature
Add DTT to appropriate concentration just before use

Warm the solution before use because it tends to solidify.

COMMENTARY

Background Information

The two most common acyl groups that modify proteins are 14-carbon myristic acid and 16-carbon palmitic acid (Fig. 14.2.2), and they occur both on different and on overlapping sets of proteins. By increasing the hydrophobicity of the protein, these fatty acid moieties can play a role in localization of the protein to the membrane and sometimes to specific types of membrane structures—e.g., glycolipid-enriched domains or caveolae (Rodgers et al., 1994). Identifying the type of acylation of a protein and determining whether the level of modification can be affected by stimuli can provide more information on the mechanisms of action of proteins involved in signaling pathways.

Fatty acids are used in labeling cells in vitro because they will diffuse across the plasma membrane and then be converted to acyl-CoA by the action of the enzyme acyl-CoA synthetase. This activated form of the fatty acid is

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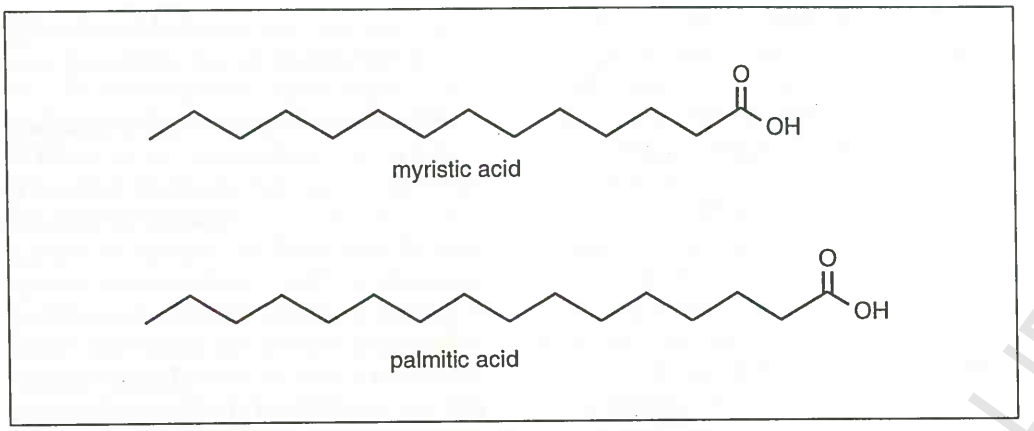

Figure 14.2.2 Structures of myristic and palmitic acids.

the substrate for protein-acyl transferases that transfer the acyl group to the protein. Tritiated fatty acids are most commonly used in biosynthetic labeling of proteins, but fluorescent analogs of fatty acids have been used to study palmitoylation of rhodopsin (Moench et al., 1994a,b), and [ω-^{125}I]iodo–fatty acids have been used to study myristoylation of v-src (Peseckis et al., 1993).

A wide variety of proteins are myristoylated, including viral structural proteins and many proteins involved in cell signaling, such as the α subunits of trimeric G proteins and the Src family of tyrosine kinases (Resh, 1994; Wedegaertner et al., 1995). Myristoylation most commonly occurs cotranslationally via an amide linkage to an NH_2-terminal glycine residue, and is an irreversible modification. Myristoylation is dependent on the removal of the initiator methionine and has been shown to occur by the time the nascent polypeptide is 100 amino acids long. Inhibitors of protein synthesis will therefore block myristoylation. The enzyme responsible for NH_2-terminal myristoylation, *N*-myristoyl transferase (NMT), was first isolated from *Saccharomyces cerevisiae*; both the yeast and human homologs have been well characterized and show different protein substrate specificities (Gordon et al., 1991). Substrate specificity of yeast NMT is determined by recognition of a sterically unhindered NH_2-terminal glycine followed by an amino acid sequence that conforms to the following criteria: an uncharged residue or proline at position 2; any amino acid at positions 3 and 4; serine (more common), alanine, or threonine at position 5; no proline at position 6; and, preferably, basic residues at positions 7 and 8. Residues C-terminal to this region appear to be important in recognition of the α subunits of trimeric G proteins and the Src family of tyrosine kinases (Glover et al., 1988; Gordon et al., 1991).

The NMT enzymatic reaction proceeds by formation of a myristoyl-CoA-enzyme complex, subsequent binding of the peptide, transfer of the myristate moiety to the peptide, release of CoA, and release of the myristoylated peptide (Rudnick et al., 1991). Several assays have been developed for this enzyme (e.g., King and Sharma, 1991; Rudnick et al., 1992; French et al., 1994). Inhibitors of myristoylation include 2-hydroxymyristic acid, which is converted in vivo to 2-hydroxymyristoyl-CoA, a potent inhibitor of NMT. Other inhibitors of acylation are fatty acid analogs and other compounds that inhibit acyl CoA synthetase and therefore block the conversion of fatty acids to acyl CoA; the latter include α-bromopalmitate (DeGrella and Light, 1980) and the triacsin family of antibiotics (Tomoda et al., 1987). The endogenous pool of fatty acid can be depleted by incubation with cerulenin, an antibiotic that inhibits de novo fatty acid biosynthesis.

Myristoylation of a protein can be necessary for its activity, e.g., the transforming activity of Src (Kamps et al., 1986). Myristoylation alone may not be sufficient for a protein to be localized to the membrane; for this further lipid modification, such as palmitoylation or cooperative interaction with protein sequences, is required (Resh, 1994).

Palmitoylation (with and without myristoylation) occurs on many signaling molecules, including rhodopsin, α-subunits of G proteins, and Src-family tyrosine kinases. Palmitoylation is a post-translational event occurring via a thioester linkage to a cysteine residue, and where it occurs with NH_2-terminal myristoylation, the myristoylation is usually a prerequisite for palmitoylation. This may be because the enzyme responsible for palmitoylation recog-

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nizes the myristoylated protein or, more likely, because myristoylation brings the protein to the correct cell location for palmitoylation to occur. Palmitoylation is also found in conjunction with isoprenoid modification at the C-terminus of proteins belonging to the Ras superfamily; it is responsible for the localization of these proteins to the membrane (Newman and Magee, 1993). This palmitoylation is dependent on prior modification of the protein by isoprenoid. G protein subunits such as the α_1 subfamily and members of the Src family of tyrosine kinases have an NH_2-terminal amino acid sequence of Met-Gly-Cys, where the initiator methionine is removed and replaced by myristate and the cysteine is palmitoylated (Resh, 1994). Palmitoylation is a reversible modification and has been shown to be dynamic in vivo, with the level of palmitoylation changing in response to various stimuli such as receptor activation, insulin, and growth factors (James and Olsen, 1989; Jochen et al., 1991; Wedegaertner et al., 1995). This phenomenon is thought to play a role in switching on or off signaling molecules by altering either the localization of the molecules or their presentation to other signaling molecules with which they interact. Depalmitoylation of proteins is catalyzed by the activity of protein-palmitoyl thioesterase (Camp and Hoffman, 1993). Protein-palmitoyl transferase has been localized to the Golgi/endoplasmic reticulum, and attempts to purify it to homogeneity have so far been unsuccessful.

Analysis of the type of fatty acid linkage present should always be performed. Post-translational myristoylation via a thioester linkage has been found in platelets (Muszbek and Laposata, 1993). The term "palmitoylation" is not strictly accurate because other long-chain fatty acids, such as stearate (18:0) and oleate (18:1), can also be thioesterified to proteins; "*S*-acylation" is now becoming more commonly used to describe this modification. The acylating activity seems to be relatively unspecific for chain length and degree of unsaturation, and utilizes acyl-CoAs partly in proportion to their abundance in the cell—hence the predominance of palmitate. This and the potential for interconversion of labeled fatty acids necessitate analysis of the chain length of the attached label. Other more complicated methods can be used to identify the type of fatty acid attached to the protein; these include gas chromatography, reversed-phase HPLC, and mass spectroscopy (Aitken, 1992). Structural and sequence analysis of the acylated protein can be performed using mass spectrometry (Aitken, 1992).

Further information about the cellular localization of acylated proteins can be found by extraction of the cell lysate in the presence of either Triton X-114 or X-100. Extraction with Triton X-114, which has the property of phase separation at 30°C, can distinguish between hydrophobic and hydrophilic proteins (Aitken, 1992). Extraction of membranes with Triton X-100 at 4°C and 37°C can identify proteins that are associated with glycolipid-enriched membranes (Rodgers et al., 1994).

Critical Parameters and Troubleshooting

Cells should be subconfluent; it is recommended that the cells be plated at two split ratios so the culture closest to 70% to 80% confluency can be selected to be used for labeling.

The pH of all solutions should be ≤7.5 in order to avoid hydrolysis of labile thioesters; the high pH of SDS-PAGE buffers does not seem to be a problem when using minigels, where the running times are relatively short. Dithiothreitol (DTT) or 2-mercaptoethanol should be used with care, as these will also cleave thioester bonds; for SDS-PAGE a maximum concentration of 20 mM DTT should be used and samples should not be boiled but incubated only 3 min at 80°C.

The use of short labeling times (especially for palmitoylation) will reduce reincorporation of the label.

The ability to detect myristoylation and/or palmitoylation of a protein using these methods will depend on the ability of the cells to take up radiolabeled compounds and incorporate them into metabolic precursors; the pool sizes of endogenous fatty acids and fatty acyl–CoA esters; the expression level of the protein-myristoyl transferase and supposed protein-palmitoyl transferase; the abundance, rate of synthesis, and turnover of the protein(s) and modification(s) of interest; and the efficiency of antibodies for immunoprecipitation.

Anticipated Results

Typically, it is possible to detect myristoylated proteins using fluorographic exposure times ranging from <1 week for high-level expression of protein in transformed cells (e.g., Lck in LSTRA cells) to 1 to 4 weeks for a well-expressed endogenous protein.

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Time Considerations

In vitro labeling experiments require 2 to 3 days for growing and labeling the cells. Harvesting the cells, preparing the lysate for SDS-PAGE (with or without prior immunoprecipitation) and SDS-PAGE require 1 to 2 days plus time for fluorography.

Linkage analysis of the labeled proteins takes 1 to 2 hr after the gel has been run. Analysis of the label in cell lysates requires 1 day to precipitate the protein, remove free label, and hydrolyze the sample. A second day is required to extract the label and perform thin-layer chromatography, and fluorography requires an overnight exposure. Analysis of the protein-bound label in gel bands takes ~3 days after fluorography (dried gel) and a similar amount of time from a wet gel, except the analysis begins after the gel has been run.

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A compilation of methods used in studying lipid modification of proteins.

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Analysis of Protein Prenylation and Carboxyl-Methylation

In the last fifteen years or so, several post-translational modifications of proteins with lipid moieties have been discovered. Analysis of fatty acylation is covered in *UNIT 14.2*. This unit describes methods for analysis of prenylation and the carboxyl-methylation that often accompanies it. The two prenoid groups that have been found attached to proteins—farnesyl (C15) and geranylgeranyl (C20)—are both derived from intermediates in the isoprenoid biosynthetic pathway that utilizes mevalonic acid. In the protocols described here, radiolabeled mevalonate is used to label these intermediates in either intact cells (see Basic Protocol 1) or in vitro translations (see Basic Protocol 2); the labeled intermediates then become incorporated into proteins. Alternatively, the preformed radioactive prenyl diphosphates can be used for in vitro translations (see Basic Protocol 2). Carboxyl-methylation of C-terminal prenylated cysteine residues often occurs subsequent to prenylation. Methods are given for radiolabeling of the methyl group with [^{3}H-methyl]-methionine, that is converted intracellularly into *S*-adenosylmethionine (see Basic Protocol 3), and for radiolabeling with preformed *S*-adenosyl[^{3}H]methionine (see Basic Protocol 4). Three recent publications (Casey, 1990; Hooper and Turner, 1992; Casey and Buss, 1995) contain a number of methods related to analysis of lipid modification of proteins.

PRENYLATION OF PROTEINS IN CULTURED CELLS

BASIC PROTOCOL 1

To radiolabel intermediates in the isoprenoid biosynthetic pathway, cultured cells are incubated with [^{3}H]mevalonic acid, which is the 6-carbon precursor to isopentenyl diphosphate. Isopentenyl diphosphate (5-carbon) is polymerized in a stepwise fashion to yield farnesyl (15-carbon) and geranylgeranyl (20-carbon) diphosphates, which are the known precursors of protein-bound prenyl groups. To maximize incorporation of exogenous [^{3}H]mevalonate, the endogenous pool of unlabeled mevalonate is usually depleted by preincubation with an inhibitor of hydroxymethyl glutaryl–coenzyme A (HMG-CoA) reductase, the enzyme responsible for production of mevalonate from hydroxymethyl glutarate. Medium containing dialyzed serum is also used to reduce the endogenous pool.

Materials

Cells
Complete tissue culture medium appropriate for the cells, 37°C
Complete tissue culture medium containing dialyzed serum, 37°C
10 mM mevinolin (see recipe)
1 μCi/μl R-[5-^{3}H]mevalonic acid (10 to 40 Ci/mmol; DuPont NEN or American Radiolabeled Chemicals)
PBS, pH 7.2 (*APPENDIX 2E*) ice-cold
1× SDS-PAGE sample buffer (*UNIT 10.1*) or RIPA lysis buffer (*UNIT 13.2*)
Nitrogen gas

Additional reagents and equipment for immunoprecipitation (*UNIT 13.2*), SDS-PAGE (*UNIT 10.1*), treating a gel with sodium salicylate (see Basic Protocol 3) or DMSO/PPO solution (*UNIT 10.2*), and fluorography (*UNIT 10.2*)

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

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1. On the day before the labeling experiment, split the cells into complete tissue culture medium.

   Set up cells at two split ratios and choose the culture closest to 70% to 80% confluency for labeling. The cultures should contain 10^6 to 10^7 cells.

2. On the day of the experiment, replace the culture medium with a minimum volume of prewarmed complete tissue culture medium containing dialyzed serum. Add 10 mM mevinolin to a final concentration of 50 µM. Preincubate the cells in this medium ≥60 min.

   Mevinolin is an inhibitor of HMG-CoA reductase. Alternative HMG-CoA reductase inhibitors are compactin/mevastatin (Sigma) and pravastatin (Pravachol, Bristol-Meyers Squibb).

3. Transfer enough 1 µCi/µl R-[5-^{3}H]mevalonic acid to label all samples at a concentration of 50 to 200 µCi/ml into a microcentrifuge tube. If necessary, remove organic solvent by exposing the tube to a gentle stream of nitrogen, but do not dry the mevalonic acid completely.

   The volume of the [^{3}H]mevalonic acid can be made up with sterile water to facilitate accurate pipetting.

   Alternatively, the organic solvent may be removed in a Speedvac evaporator.

   The mevalonic acid must not be heated above room temperature because it is unstable.

4. Add the partially dried [^{3}H]mevalonic acid at the desired concentration to the culture medium. Incubate up to 24 hr.

   The most appropriate concentration of label and duration of incubation should be experimentally determined for each cell type.

5. Place the cells on ice and aspirate the medium. Wash the cells twice with ice-cold PBS.

   CAUTION*: Radioactive medium and washes must be disposed of appropriately.*

6. Lyse cells in the appropriate solution for further processing—e.g., for a 50-mm dish containing 4×10^6 cells, add 0.2 ml 1× SDS-PAGE sample buffer (*UNIT 10.1*) or 1 ml RIPA lysis buffer for immunoprecipitation (*UNIT 13.2*).

   Use the entire lysate for immunoprecipitation.

7. Analyze the lysate or immunoprecipitate by SDS-PAGE (*UNIT 10.1*).

   Use 25 µl of the whole cell lysate for a minigel or 50 µl for a standard gel; load all of the immunoprecipitate after solubilizing it in 25 or 50 µl of 1× SDS-PAGE sample buffer.

8. After SDS-PAGE, treat the gel with sodium salicylate (see Basic Protocol 3, step 6) or DMSO/PPO solution (*UNIT 10.2*; Magee et al., 1995) and fluorograph the gels at –70°C using preflashed film.

   Typical exposure times are 1 week to 1 month, although exposures up to 3 months may sometimes be required (see Time Considerations).

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PRENYLATION OF PROTEINS DURING IN VITRO TRANSLATION

Commercially available reticulocyte lysates contain protein:prenyl transferases, so proteins can be labeled during in vitro translation of mRNA using [^{3}H]mevalonic acid (Hancock et al., 1991; Newman et al., 1992; Giannakouros et al., 1993), which is converted to prenyl diphosphates in situ. When [^{3}H]mevalonic acid is used, the identity of the incorporated label as farnesyl or geranylgeranyl must be experimentally determined by cleavage of the label followed by chain-length analysis using the method described by Farnsworth et al. (1990). In the author's experience, commercially available reticulocyte lysates contain little endogenous mevalonate, so prenylation is highly dependent on added precursors. Alternatively, presynthesized [^{3}H]prenyl diphosphates can be used; this allows an assignment of the protein-bound prenoid by labeling duplicate translations with [^{3}H]farnesyl diphosphate or [^{3}H]geranylgeranyl diphosphate. Incorporation of only one precursor into the protein unambiguously identifies the prenoid substituent. Incorporation of both suggests either that farnesyl diphosphate has been converted to geranylgeranyl diphosphate in the translation mix, or that the protein under investigation genuinely contains both prenoids. This question must be resolved by chain-length analysis (Farnsworth et al., 1990).

Materials

Nuclease-treated rabbit reticulocyte lysate (Promega)
Methionine-free amino acid mix (Promega)
100 mM L-methionine
1 µCi/µl R-[5-^{3}H]mevalonic acid (10 to 40 Ci/mmol; DuPont NEN or American Radiolabeled Chemicals)
mRNA: e.g., in vitro transcribed RNA derived from cDNA cloned into an appropriate vector such as SP64T, pGEMp9Z(f-), or pBluescript

6× SDS sample buffer (*UNIT 10.1*)
Trichloroacetic acid
Nitrogen gas
30°C water bath

Additional reagents and equipment for immunoprecipitation (*UNIT 13.2*; optional), SDS-PAGE (*UNIT 10.1*), and fluorography (*UNIT 10.2*)

1. Using nuclease-treated rabbit reticulocyte lysate and methionine-free amino acid mix and following manufacturer's instructions, prepare a master mix containing 25 µl translation mix for each mRNA to be translated and a negative control tube on ice.
2. Add 0.25 µl of 100 mM L-methionine (final concentration 1 mM) per reaction.
3. For each sample, aliquot 20 µCi R-[5-^{3}H]mevalonic acid into a microcentrifuge tube. Remove the solvent under a stream of nitrogen gas.

   Alternatively, use 2.5 µCi of [1-^{3}H]farnesyl diphosphate or [1-^{3}H]geranylgeranyl diphosphate (30 to 60 Ci/mmol; American Radiolabeled Chemicals or DuPont NEN) per sample and remove the solvent under a stream of nitrogen gas.
4. Add the translation master mix to the tube containing labeled precursor. Incubate 10 min on ice.
5. Aliquot 25 µl of the mix into separate microcentrifuge tubes for each sample.
6. Start the translation by adding mRNA (e.g., 1 µg of in vitro–transcribed RNA). Incubate 90 min in a 30°C water bath.

   The optimum RNA concentration and duration of translation must be determined for each batch of reticulocyte lysate and each mRNA.

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7. At the end of the translation reaction, stop the reaction by adding 5 µl of 6× SDS-PAGE sample buffer or 1 ml RIPA lysis buffer, and process the samples for SDS-PAGE (see Basic Protocol 1 and *UNIT 10.1*), or immunoprecipitation (see Basic Protocol 1 and *UNIT 13.2*). Load the entire sample on the gel. Alternatively, add trichloroacetic acid to a final concentration of 15% (w/v) and process for prenoid analysis (Farnsworth et al., 1990).

BASIC PROTOCOL 3

CARBOXYL-METHYLATION OF PROTEINS IN CULTURED CELLS

Prenylation at C-terminal CXXX and CXC motifs is followed by carboxyl-methylation of the α-carboxyl group. This is performed by a membrane-associated methyl transferase that uses the universal methyl group donor *S*-adenosylmethionine (SAM). The methyl group of SAM can be radiolabeled in vitro by [^{3}H-methyl]methionine, and it is then transferred onto the α-carboxyl group as an alkali-labile ester. This can be distinguished from alkali-stable methylations such as lysyl N-methylation by treating the sample with sodium hydroxide, which releases [^{3}H]methanol. The released volatile [^{3}H]methanol is collected by distillation into scintillation fluid and counted (Chelsky et al., 1985; Gutierrez et al., 1989). Labeled methionine will also be incorporated into the peptide backbone and is alkali-stable, thus providing an internal standard that can be used to calculate the stoichiometry of carboxyl-methylation. The length of the labeling incubation required to get an acceptable signal depends inversely on the expression level of the protein under study.

Materials

Cells
Complete tissue culture medium (CM) appropriate for the cells
Methionine-free tissue culture medium containing dialyzed serum (MFM) *or* 5% (v/v) complete culture medium with dialyzed serum (MFM-5%CM)
1 µCi/µl L-[^{3}H-methyl]methionine (~80 Ci/mmol, Amersham)
PBS, pH 7.2 (*APPENDIX 2E*), ice-cold
1× SDS-PAGE sample buffer (*UNIT 10.1*) or RIPA lysis buffer (*UNIT 13.2*)
1 M sodium salicylate, or other water-soluble fluor (e.g., Enlightening, DuPont NEN, or Amplify, Amersham)
Radioactive ink (see recipe)
1 M NaOH
1 M HCl
Warm room or incubator at 37°C

Additional reagents and equipment for immunoprecipitation (*UNIT 13.2*), SDS-PAGE (*UNIT 10.1*), and fluorography (*UNIT 10.2*)

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Label the cells

1. On the day before the labeling experiment, split the cells into fresh complete tissue culture medium.

   Set up cells at two split ratios and choose the culture closest to 70% to 80% confluency for labeling. The cultures should contain 10^6 to 10^7 cells.

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For labeling times ≤8 hr

2a. On the day of the experiment, replace the culture medium with the minimum volume MFM, e.g., 3 ml for a 100-mm tissue culture dish. Incubate 1 hr.

3a. Add 200 µCi [^{3}H-methyl]methionine per milliliter MFM. Incubate up to 8 hr.

For labeling times of 8 to 24 hr

2b. On the day of the experiment, replace the culture medium with the minimum volume MFM-5%CM, e.g., 3 ml for a a 100-mm tissue culture dish. Incubate 1 hr.

3b. Add 50 µCi [^{3}H-methyl]methionine per milliliter MFM-5%CM. Incubate 8 to 24 hr.

4. After labeling is complete, aspirate the medium and wash the cells twice with ice-cold PBS.

5. Lyse in the appropriate solution for further processing—e.g., for a 50-mm dish containing 4×10^6 cells, add 0.2 ml 1× SDS-PAGE sample buffer (*UNIT 10.1*) or 1 ml RIPA lysis buffer for immunoprecipitation (*UNIT 13.2*).

Separate and fluorograph the samples

6. Analyze the immunoprecipitate or lysate by SDS-PAGE (*UNIT 10.1*).

   Use 25 µl whole-cell lysate for a minigel or 50 µl for a standard gel. Use the whole immunoprecipitate solubilized in 25 or 50 µl of 1× SDS-PAGE sample buffer.

   The α-carboxyl-methyl esters are stable at the moderately alkaline pH of the standard Laemmli gel system, whereas methyl esters of the side-chain carboxyls of aspartate and glutamate are quite labile.

7. Soak the gel 20 min in 1 M sodium salicylate. Dry the gel immediately at 60°C on a backing of filter paper.

   The gel must not be dried at too high a temperature.

8. Using radioactive ink, mark the filter paper to note the alignment of the gel. Fluorograph the gel at –70°C, using preflashed film. Expose the film for 1 week to 1 month.

   An inexpensive radioactive ink can made by washing out old radiolabel vials with a small volume (e.g., 0.1 ml) of an aqueous solution containing 0.01% (w/v) bromphenol blue.

9. Using the alignment marks, align the film exactly with the gel. Excise the radioactive band(s) of interest with a scalpel.

Release the alkali-labile label

10. Place each gel piece into a separate 1.5-ml microcentrifuge tube. Carefully lower the open tube into a scintillation vial that contains enough scintillation fluid to come about half way up the side of the tube.

11. Add enough 1 M NaOH into the tube to cover the gel piece (usually 100 to 200 µl). Immediately cap the scintillation vial, leaving the tube open inside. Incubate overnight in a 37°C warm room or 37°C incubator.

    During this incubation, radioactive methanol is hydrolytically released and distilled into the scintillation fluid.

12. The next day, uncap the vial and carefully remove the microcentrifuge tube, taking care not to spill any of the contents into the vial. Recap the vial.

    This scintillation vial contains the alkali-labile [^{3}H]methanol.

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Analyze the sample for alkali-stable label

13. Neutralize the NaOH in the microcentrifuge tube by adding an equal volume of 1 M HCl. Transfer the contents of the tube to a new scintillation vial.

    This scintillation vial contains the alkali-stable [^{3}H]label which represents [^{3}H]methionine incorporated into the peptide backbone. However, alkali-stable methyl groups cannot be distinguished from backbone methionine in this way, so it is essential to have good reason to believe that other methyl groups are not present before alkali-stable counts can be used to calculate stoichiometry of carboxyl-methylation.

14. Add a volume of scintillation fluid to this vial equal to that used for the alkali-labile sample (step 12).

15. Count both samples in a scintillation counter using the tritium channel.

Calculate the methylation stoichiometry

16. Calculate the methylation stoichiometry as:

$$\text{stoichiometry} = \frac{\text{alkali–labile cpm} \times \text{number of methionine residues in the protein}}{\text{alkali–stable cpm.}}$$

    This calculation can only be made if the methionine content of the protein is known. Also the possibility that the initiator methionine might be removed must be taken into account. This can be predicted from the primary sequence (Aitken, 1992).

BASIC PROTOCOL 4

CARBOXYL-METHYLATION DURING OR AFTER IN VITRO TRANSLATION

Carboxyl-methylation can also be carried out during or after in vitro translation of mRNA (Newman et al., 1992; Giannakouros et al., 1993). Unlabeled mevalonic acid must be included in the reaction because carboxyl-methylation obligatorily follows prenylation. A source of the membrane-bound methyltransferase must also be added—either a crude 100,000 × *g* membrane pellet from cultured cells or commercially available dog pancreatic microsomes will suffice. These membranes also contain the protease required to remove the XXX residues from the C-terminal CXXX motif of prenylated proteins, which must occur before carboxyl-methylation can take place. Translation can be performed in the presence of preformed *S*-adenosyl[^{3}H-methyl]methionine and membranes, or alternatively, the radiolabel and membranes can be added after translation has been completed. Methionine must be added to support translation, because the manufacturer's amino acid mix lacks it.

Materials

Nuclease-treated rabbit reticulocyte lysate (Promega)
Methionine-free amino acid mix (Promega)
100 mM L-methionine
100 mM mevalonic acid (see recipe)
1 μCi/μl *S*-adenosyl[^{3}H-methyl]methionine (5 to 15 Ci/mmol, Amersham or ICN Biomedicals)
Microsomal membranes: 100,000 × *g* pellet from cultured cells or canine pancreatic microsomal membranes (Promega; store at –70°C and thaw on ice immediately before use)
mRNA: e.g., in vitro transcribed RNA derived from cDNA cloned into an appropriate vector such as SP64T, pGEMp9Z(f-), or pBluescript

Nitrogen gas
Water bath at 30°C

Additional reagents and equipment for immunoprecipitation (*UNIT 13.2*), SDS-PAGE (*UNIT 10.2*), and fluorography (*UNIT 10.2*)

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1. Using nuclease-treated rabbit reticulocyte lysate and methionine-free amino acid mix and following manufacturer's instructions, prepare a master mix containing 25 µl of translation mix for each mRNA to be translated and a negative control tube on ice.
2. Add 0.25 µl of 100 mM L-methionine (final concentration 1 mM) and 1.25 µl of 100 mM mevalonic acid (final concentration 5 mM) for each 25 µl of master mix.
3. For each reaction, aliquot 30 µCi *S*-adenosyl[^{3}H-methyl]methionine into a microcentrifuge tube. Remove the solvent under a stream of nitrogen.

   Alternatively, the solvent can be removed in a Speedvac evaporator.
4. Add the translation master mix to the tube that contains labeled precursor. Incubate 10 min on ice.
5. Add 3.6 equivalents of microsomal membranes for each 25 µl of master mix to the tube.

   Either total microsomal membranes (the 100,000 × g pellet) from cultured cells or tissue or a commercial preparation of canine pancreatic microsomal membranes can be used. The optimum amount of membrane to use will have to be determined experimentally, but a good starting point is 3.6 equivalents (Promega) per 25 µl of translation mix.

   One equivalent of microsomal membranes is that amount of membranes required to cleave the signal sequence of preprolactin from 50% of the translation products, as measured by a shift in mobility on SDS-PAGE gels (from Promega Canine Pancreatic Microsomal Membranes information sheet).
6. Aliquot 25 µl of the master mix into separate microcentrifuge tubes for each sample.
7. Start the translation by adding mRNA (e.g., 1 µg of in vitro transcribed RNA). Incubate the tubes 90 min in a 30°C water bath.

   The optimum RNA concentration and duration of translation must be determined for each batch of reticulocyte lysate and each mRNA.

   Alternatively, in vitro translation can be performed in the absence of label and membranes. In that case, add 30 µCi label and 3.6 equivalents of microsomal membranes to each tube after translation and incubate an additional 2 hr at 30°C.
8. At the end of the translation reaction, process the samples for SDS-PAGE (see *UNIT 10.1*) or immunoprecipitation (see *UNIT 13.2*) and fluorography (*UNIT 10.2*).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Mevalonic acid, 100 mM

Hydrolyze mevalonic acid lactone (Sigma) to the free acid (Kita et al., 1980) by dissolving 30 mg mevalonic acid lactone in 1.8 ml ethanol at 55°C. Add 0.9 ml of 0.6 M NaOH and 18 ml water. Incubate 30 min at room temperature. Adjust to pH 8 with HCl and dilute to 100 mM. Store indefinitely at –20°C.

Mevinolin, 10 mM

Dissolve 90 mg mevinolin in 1.8 ml ethanol at 55°C. Add 0.9 ml of 0.6 M NaOH and 18 ml distilled water. Incubate 30 min at room temperature. Adjust to pH 8 with HCl and dilute to 10 mM. Store indefinitely at –20°C in 500-µl aliquots.

Mevinolin, also called Lovastatin and Mevacore, is available from Merck & Co. Inc. or Biomol Research Labs (see SUPPLIERS APPENDIX; Alberts, 1988). It is supplied as the

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lactone and must be hydrolyzed to the free acid as described in this recipe (Kita et al., 1980).

Radioactive ink

An inexpensive radioactive ink can be made by washing out old radiolabel vials with a small volume (~0.1 ml) of an aqueous solution containing 0.01% (w/v) bromphenol blue.

COMMENTARY

Background Information

Prenylation of proteins is a widespread post-translational modification that occurs at C-terminal motifs of the type CXXX, CXC, or CC (where C is cysteine and X is any amino acid). Recent reviews give a thorough description of this field (Clarke, 1992; Giannakouros et al., 1993). Either the 15-carbon isoprenoid farnesol or the 20-carbon isoprenoid geranylgeraniol (see Fig. 14.3.1) can be added, depending on the exact sequence at the C-terminus. $CX_1X_2X_3$ motifs where X_3 is a small residue (e.g., methionine, serine, or alanine), are farnesylated; whereas if X_3 is large and hydrophobic (e.g., leucine or phenylalanine), they are geranylgeranylated. The nature of the X_1 and X_2 residues also influences the ability of the motif to become prenylated such that some apparent CXXX sequences are not modified. The CXC and CC motifs are also geranylgeranylated on both cysteines. The prenoid moiety is attached by a thioether linkage to the sulphydryl group of cysteine; this is an extremely stable linkage that is irreversible in vivo, but it can be chemically cleaved in vitro to allow analysis of the chain length of the attached isoprenoid (Farnsworth et al., 1990).

Prenylation occurs on otherwise cytosolic soluble proteins and is catalysed by a ubiquitous family of at least three soluble heterodimeric enzymes: farnesyl transferase, geranylgeranyl transferase I (active on CXXX motifs) and geranylgeranyl transferase II (active on CXC and CC motifs). In addition, geranylgeranyl transferase II has a third associated subunit called Rab escort protein that is required for activity. These enzymes utilize the activated diphosphates of farnesol and geranylgeraniol that are normal intermediates in the isoprenoid biosynthetic pathway, that leads to many important end products such as steroids, ubiquinone, and dolichols (Goldstein and Brown, 1990). Mevalonic acid is the precursor of these polyisoprenyl diphosphates and is readily taken up by most cells in culture. Hence radioactive mevalonate can be used to radiolabel prenyl chains biosynthetically. To maximize incorporation, endogenous mevalonate is usually depleted by preincubation of cells with an inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme responsible for mevalonate synthesis. Both the ability of cells to take up exogenous mevalonate and the sensitivity to HMG-CoA reductase inhibitors can vary enormously and needs to be experimentally tested (e.g., the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*; Giannakouros et al., 1992).

Subsequent to and dependent on prenylation, other modifications can take place. CXXX motifs are proteolyzed to remove the XXX residues by a membrane-associated en-

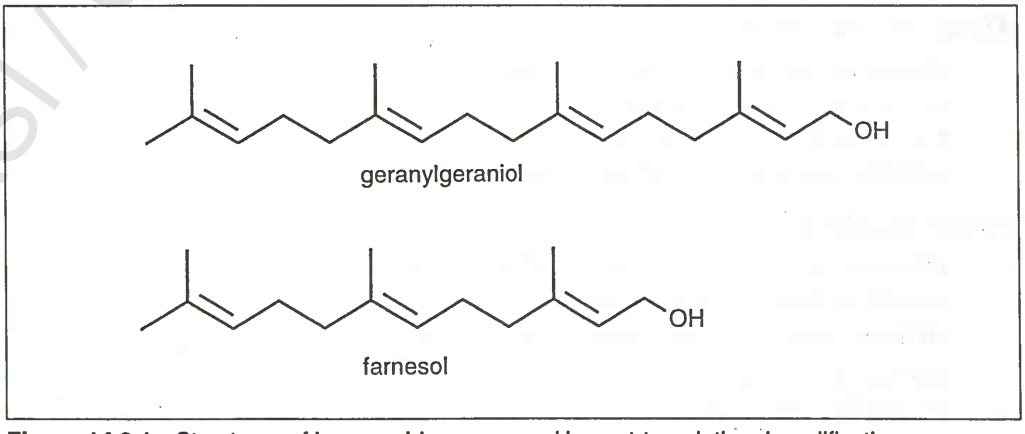

Figure 14.3.1 Structures of isoprenoid groups used in post-translational modification.

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doprotease; then they are carboxyl-methylated on the free C-terminal α-carboxyl by a membrane-bound carboxyl-methyl transferase. This methyl ester group is chemically relatively labile; it is sensitive to mild alkali, although it is not as labile as the side-chain methyl esters of aspartate and glutamate, which can hydrolyze even during electrophoresis in mildly alkaline buffers such as are used in the standard Laemmli SDS-PAGE system (Laemmli, 1970). This lability is used experimentally as a test for the C-terminal α-carboxyl-methyl ester (Chelsky et al., 1985; Gutierrez et al., 1989). CXC motifs become doubly geranylgeranylated and are therefore also carboxyl-methylated because the methyl transferase recognizes a C-terminal prenylated cysteine. CC motifs, on the other hand, are not carboxyl-methylated. Many prenylated proteins also become palmitoylated *S*-acylated on nearby upstream cysteines by a membrane-bound palmitoyl transferase that is poorly characterized. Palmitoylation is discussed in *UNIT 14.2*.

The primary function of prenylation is in membrane localization of substrate proteins that would otherwise be cytosolic because they are usually lacking in hydrophobic peptide sequences (Parenti and Magee, 1995). To perform this localization function, prenyl groups generally act in cooperation with themselves (e.g., for the doubly geranylgeranylated CXC proteins), with other modifications such palmitate moieties, or with peptide sequences. For example, the prenylated CXXX sequences of Ras proteins must cooperate with palmitoylation sites (in the case of H-ras, N-ras and K[A]-ras) or a polybasic region (as in K[B]-ras), which probably interacts with acidic membrane phospholipids (Hancock et al., 1991; Newman and Magee, 1993). These cooperating signals specify not only general membrane binding, as might be expected for a hydrophobic lipid moiety, but also targeting to specific subcellular destinations. The mechanism of this intracellular targeting is at present obscure.

Membrane association of prenylated proteins is frequently reversible in vivo. This is achieved in several ways (Newman et al., 1992). Both palmitoylation and carboxyl-methylation can be dynamic in vivo, thus allowing modulation of the hydrophobicity and/or charge of the C-terminus. Phosphorylation of residues near the C-terminus will change the charge, reducing the interaction with acidic phospholipid head groups. Thirdly, many prenylated proteins are removed from membranes by sequestration of their prenylated domains by binding proteins that mask the hydrophobic group and form soluble 1:1 complexes. These mechanisms allow prenylated proteins to move between subcellular sites, which is often an essential part of their function.

Critical Parameters and Troubleshooting

All of the labeling protocols in this unit that use cultured cells perform optimally when subconfluent cells are used. Certainly cells that are fully confluent should not be used. To ensure that cells at an appropriate density are available, it is helpful set up cultures at two different split ratios and use the ones that are most suitable. Occasionally a cell line is encountered that labels very poorly. Increasing the amount of radiolabel and/or the duration of labeling may lead to a satisfactory result. In the case of [^{3}H]mevalonic acid labeling, the amount of mevinolin used and the duration of pretreatment can be increased. [^{14}C]mevalonic acid has been used by some workers for radiolabeling prenyl moieties. Although tritium has a lower intrinsic detectability than ^{14}C due to its weaker emission, the author nevertheless finds ^{3}H is more sensitive because tritiated compounds can generally be obtained at much higher specific activities than ^{14}C derivatives, and that more than compensates for the lower emission. For [^{3}H-methyl]methionine labeling, it may help to reduce the concentration of unlabeled methionine in the medium to zero and to increase the duration of pretreatment.

It is always useful to include an aliquot of the total cell lysate on the SDS-PAGE gel to assess the efficiency of labeling. Low levels of tritium require the highest sensitivity of fluorography for most rapid detection. The author has found that treating the gel with 2,5-diphenyloxazole in dimethylsulfoxide (DMSO/-PPO solution, *UNIT 10.2*) is the most sensitive method (Laskey, 1980; Magee et al., 1995). However, this method cannot be used if further processing of the gel for prenyl group analysis or alkaline release of carboxyl-methyl groups is to be performed. In these cases, fluorography is best performed after treating the gel with the water-soluble fluor salicylic acid (Chamberlain, 1979) or a commercial equivalent; this method is approximately half as sensitive.

Mevalonic acid and mevinolin tend to cyclize to form the lactone. If this is suspected (e.g., as indicated by a fall-off in labeling efficiency), the lactone can be hydrolyzed by the method of Kita et al. (1980) as described in the

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recipes (see Reagents and Solutions). Lipid substrates should never be dried to completion in plastic tubes because they are often irreversibly adsorbed to the surface.

The ability to detect prenylation and/or carboxyl-methylation of any given protein using these methods will depend on a number of factors: the ability of the cells to take up radiolabeled compounds and incorporate them into metabolic precursors; the sensitivity of the cells to hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors; the pool sizes of endogenous mevalonate, prenyl diphosphates, and *S*-adenosylmethionine (SAM); the expression level of the protein prenyl transferases and carboxyl-methyl transferase; the abundance, rate of synthesis, and turnover of the protein(s) and modification(s) of interest; and for immunoprecipitation, the efficiency of the antibodies.

Anticipated Results

Typically it is possible to detect prenylated proteins using fluorographic exposure times of one to a few days for transfected cDNAs expressed at high levels, e.g., in COS cells, or in vitro translated mRNAs, 2 or 3 weeks for well-expressed endogenous proteins, or up to 3 months for endogenous proteins expressed at low levels or in cells that incorporate mevalonate poorly.

Similar exposure times are required for carboxyl-methylated proteins. However, because much of the radiolabel incorporated into these proteins is in the form of backbone methionine, the exposure time to detect the protein by fluorography may not be a good guide to the sensitivity of detection for alkali-labile methyl groups. This can be predicted if the primary sequence of the protein and therefore, its methionine content is known; it clearly becomes more of a problem with larger proteins and those with many methionines.

Time Considerations

Labeling experiments using cultured cells typically require ~3 days to grow and label the cells. Harvesting and analyzing the cells by SDS-PAGE (with or without immunoprecipitation) take an additional 2 days, plus the time required for fluorography. After the fluorogram is developed, it takes 2 to 4 hr plus an overnight incubation to perform the analysis for alkali-labile and alkali-stable label, and the samples can be counted the next day.

The in vitro translation protocols typically take a single full day for translation and labeling and an additional day if immunoprecipitation is performed. The time required to complete the analysis is the same as that for the experiments using cultured cells.

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CHEMICAL MODIFICATION OF PROTEINS

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CHAPTER 15
Chemical Modification of Proteins

INTRODUCTION

Chemical modifications of reactive amino acid side chains on proteins have many applications in modern protein science. Common applications include: fluorescent or radioactive tagging of macromolecules; identifying the subcellular localization of a specific protein, i.e., cell surface, bilayer imbedded, or internal; coupling the protein to another component including another protein, nucleic acids, or a solid support; and as a aid in structural analyses (Chapter 11) such as amino acid analysis, sequencing, peptide mapping and mass spectrometry. Another historical application included the use of chemical reagents for structure/function applications, such as identification of residues in enzyme active sites. Over the past decade many, but not all, of these latter approaches have been replaced by site-directed mutagenesis analyses. However, despite the impressive power of mutagenesis methods, it is often most productive to utilize both methods in concert—i.e., initial chemical modification studies can be used to identify likely residues involved in a particular biological function, and mutagenesis studies are then used to confirm and extend these initial observations.

The most common chemical modifications target the most reactive amino acid side chains and are primarily oxidations, reductions, and nucleophilic or electrophilic substitutions. Chemical reagents that react with side chain amines or carboxyl groups will also react with the terminal amino and carboxyl groups of a protein, respectively, unless these groups were already modified in vivo. Terminal amino and carboxyl groups of a protein are, on average, more reactive than side-chain amino and carboxyl groups, and this difference can in theory be exploited to specifically label these terminal functional groups. Such specific, single-site labeling or attachment would be very useful for a range of applications, including solid phase sequencing and directional, defined attachment of proteins for affinity chromatography. In practice, selective reactivity of the terminal residue is rarely feasible. Achieving selective end-group modification requires empirical determination of appropriate reaction conditions, which must then be rigorously controlled. However, a more important limitation is that the microenvironments of individual reactive side chains can vary widely and affect reactivity to the point where some side chains are more reactive than the terminal residue.

One of the most reactive and most frequently modified amino acid side chains is the SH group of cysteine (*UNIT 15.1*). Cysteines occur in proteins either in the reduced state (cysteine or SH form) or in the oxidized state, where a disulfide bond is formed between two cysteines (cystine or -S-S- form). Disulfide bonds can be between two cysteines on the same polypeptide chain or between two different polypeptides. They are major stabilizing components of many folded proteins, especially proteins normally found in an oxidizing environment such as is typically found on the exterior of cells. In most proteins, cysteines occur relatively infrequently and are often useful sites to either tag a protein or to link it to a matrix, e.g., when crosslinking a protein to a chromatography matrix to produce an affinity column or to couple the protein to a biosensor chip for studying macromolecular interactions. Reactions using cysteine-specific reagents can also be used to distinguish between cysteine on the surface of a protein (no reduction, no denaturants), buried cysteines (denaturants, no reduction) and residues in disulfide bonds (reduction and denaturants). Finally, because cysteines are very reactive, it is necessary to convert

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them to more stable derivatives prior to amino acid or sequence analyses if identification of cysteines is desired.

Future supplements will provide protocols for derivatization of lysines and specific protocols for tagging proteins with biotin either through lysines or other reactive side chains. Labeling proteins with biotin has an especially wide range of applications in modern protein science. Later supplements will deal with reactions using carboxyl groups and other reactive side chains.

David W. Speicher

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Modification of Cysteine

This unit describes a number of methods for modifying cysteine residues of proteins and peptides by reduction and alkylation procedures. These methods vary in duration, concentration, denaturant, and reductant, but they are basically similar in approach. They consist of four basic steps: (1) preparation of the sample (i.e., buffering, denaturation, and/or reduction); (2) reaction with excess alkylating reagent; (3) quenching or removing unreacted alkylating reagent; and (4) removal of salts and reaction by-products. When pH and duration of the reaction are controlled, the modification is normally very specific for cysteinyl residues. In solution, the reaction is usually carried out under denaturing conditions, in either 6 M guanidine·HCl or 8 M urea, to optimize accessibility of cysteine side chains that may be protected from reagent access by the structure of the native protein. When denaturation is desired, the denaturing reagents should be added to the sample prior to reduction and/or addition of modifying reagent. Finally, most protocols can be adjusted to accommodate any amount of protein from the low microgram to milligram range.

A general procedure for alkylation of cysteine residues in a protein of known size and composition with haloacyl reagents or *N*-ethylmaleimide (NEM) is presented in Basic Protocol 1. Alternate Protocols 1 and 2 present similar procedures for use when the size and composition are not known and when only very small amounts of protein are available. Alkylations that introduce amino groups using bromopropylamine and *N*-(iodoethyl)-trifluoroacetamide are presented in Basic Protocols 2 and 3. Two procedures that are often used for subsequent sequence analysis of the protein, alkylation with 4-vinylpyridine and acrylamide, are described in Basic Protocols 4 and 5. In addition, a specialized procedure for 4-vinylpyridine alkylation of protein that has been adsorbed onto a sequencing membrane is presented in Basic Protocol 9. Reversible modification of cysteine residues by way of sulfitolysis is described in Basic Protocol 6, and oxidation with performic acid for amino acid compositional analysis is described in Basic Protocol 7. Gentle oxidation of cysteine residues to disulfides by exposure to air is described in Basic Protocol 8. Support Protocols are included for recrystallization of iodoacetic acid (Support Protocol 1), colorimetric detection of free sulfhydryls (Support Protocol 2), and desalting of modified samples (Support Protocol 3).

STRATEGIC PLANNING

Modification of cysteine side chains in proteins and peptides is usually done for one or more of three main reasons: (1) to break disulfide bonds and block their reformation, (2) to tag the cysteine residue with an adduct that will assist in its identification or quantitation in subsequent analytical techniques, and (3) to introduce a reporter group, such as a fluorescent label, into a protein or peptide.

The reagents most commonly used for modification of cysteine are reagents susceptible to nucleophilic attack by the cysteine sulfur (including reactive halogen compounds and compounds containing reactive double bonds) and oxidizing agents. Strong oxidizers, such as performic acid, convert thiols to sulfonic acid groups; weak oxidizers, such as oxygen, convert thiols to disulfides. Cysteine residues can also form mixed disulfides with other thiol reagents (see Background Information). Mixed disulfide formation is, in fact, the basis for thiol reagent–induced reduction of disulfide bonds, which is often the first step in cysteine modification procedures. Reaction of cysteine with reactive halogen compounds and reactive double bonds requires the presence of the free thiolate anion. Thus, any disulfides originally present must first be reduced to free thiols unless the experiment is designed to restrict modification only to preexisting thiols.

The most common method for cleaving disulfide bonds is by reduction via a disulfide interchange between the protein cysteine disulfide and a sulfhydryl group of the reducing

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Table 15.1.1 Common Reagents for Modification of Cysteine

Reagent	Molecular weight	Reaction product	Applications[a]	Comments
Iodoacetic acid	186	*S*-carboxymethyl cysteine	General	Adduct not completely stable to acid hydrolysis; introduces negative charge
Iodoacetamide	185	*S*-carboxamidomethyl cysteine	General	Converted to *S*-carboxymethyl cysteine upon acid hydrolysis (see iodoacetic acid)
5-I-AEDANS[b]	434	*S*-AEDANS-cysteine	Introduces fluorescent label	Does not interfere with Edman sequence analysis
N-ethylmaleimide	125	*S*-(ethylsuccinimido)-cysteine	General	Membrane permeable; can also react with amino groups and histidine; adduct decomposes to *S*-succinyl cysteine and ethylamine upon acid hydrolysis
3-Bromopropylamine	138	*S*-aminopropyl cysteine	Sequencing and amino acid analysis	Adduct stable and well resolved in sequencing and amino acid analysis; introduces a positive charge
N-(iodoethyl)-trifluoroacetamide	267	*S*-(2-aminoethyl)-cysteine	Introduces trypsin cleavage site	Introduces a positive charge
4-Vinylpyridine	105	*S*-pyridylethyl cysteine	Sequencing	Introduces a positive charge
Acrylamide	71	Cysteine-*S*-β-propion-amide	Sequencing	PTH-derivative is well resolved
Sodium sulfite	126	*S*-sulfocysteine	Reversible modification	Introduces negative charge; adduct stable only at neutral and acidic pH
Performic acid	—	Cysteine sulfonic acid (cysteic acid)	Amino acid analysis	Quantitation of Cys in amino acid analysis; destroys His, Tyr, Ser, Thr, Met, Trp; introduces negative charge
Air (oxygen)	—	Cystine	Formation of disulfide bonds	Slow

[a]The most frequent use of each is listed, but reagents are not necessarily restricted to this use alone.

[b]*N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine.

agent. Historically, 2-mercaptoethanol (2-ME) was used extensively, but its popularity has been eclipsed by the more efficient dithiothreitol (DTT). The efficiency of DTT is the result of the thermodynamically favored formation of an intramolecular disulfide ring structure within the reagent, which greatly shifts the equilibrium of the reaction toward complete reduction of the protein. Practically, this means that only slight excesses of DTT are necessary to achieve complete reduction; this can be meaningful for reactions using an expensive or radioactive alkylating agent. Aesthetically, even though DTT has a sulfurous odor, it does not compare with the volatile stench of 2-ME.

In the case of proteins, where secondary and tertiary structure may interfere with reagent access to the cysteine side chains, modification reactions are usually carried out in the presence of a protein denaturant such as 6 M guanidine·HCl or 8 M urea. In general, these denaturants can be used interchangeably and selection is often a matter of personal preference. However, urea solutions produce cyanate ions, which can carbamylate amino groups in proteins and peptides and thus block them to subsequent sequence analysis. If urea is used, it should always be treated with a mixed-bed ion-exchange resin to remove the cyanate just prior to its use. In addition, urea should be removed from the protein as

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soon as possible after the completion of the reaction. Of course, if carbamylation of free amino groups is not a concern, this precaution can be omitted. Also, the choice between urea and guanidine·HCl may be influenced by the choice of protease that may subsequently be used on the modified protein. This is particularly an issue when modifying very low quantities of protein with the intent to subsequently perform a proteolytic digestion. Certain proteases are active in lower concentrations of either urea or guanidine, so it may not be necessary to completely remove the denaturant. Instead, a simple dilution may be all that is necessary to proceed (see *UNIT 11.1*). Alternatively, cysteine modifications can be done with little or no denaturation to study free sulfhydryl content or the accessibility of free sulfhydryls in the native form of the protein.

The choice of which reagent to use or which protocol to follow may depend to some extent on the purpose of the modification. For instance, the intent may be to modify cysteine to stabilize it specifically for amino acid compositional analysis or sequence analysis. Some cysteine adducts are more suitable than others for this purpose; Table 15.1.1 lists those that have been used most successfully. Alternatively, the intent may simply be to prevent disulfide bond reformation for peptide separation after proteolysis, in which case most reagents are suitable. When working with very low levels of protein, volume and sample manipulation must be kept to a minimum to avoid losses in handling. The protocols presented here are general procedures that can be adapted to a number of specific uses. All of them are very simple and easy to perform. Notes in the individual protocols indicate where one offers some advantage over another for a particular use (also see Critical Parameters and Troubleshooting and Table 15.1.1). A number of specialized protocols involving cysteine modification in conjunction with a specific application are presented elsewhere in this manual. Reduction and alkylation of peptides in digest mixtures is discussed in *UNIT 11.1*, and reduction and alkylation of proteins in acrylamide gels prior to digestion is presented in *UNIT 11.3*.

ALKYLATION OF A PROTEIN OF KNOWN SIZE AND COMPOSITION WITH HALOACYL REAGENTS OR *N*-ETHYLMALEIMIDE

BASIC PROTOCOL 1

Reaction of the sulfhydryl group of cysteine with α-haloacids or their amide derivatives—iodoacetic acid, iodoacetamide, and *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (known as IAEDANS)—is historically the most common method used to alkylate cysteine. Use of iodo compounds is typical because iodide is a better leaving group (i.e., has faster reaction times) than the other halides. The choice between iodoacetic acid or iodoacetamide may be dictated by what subsequent studies are to be performed and, in some cases, the accessibility of the cysteine residues in the protein being modified (Gerwin, 1967). For instance, if the cysteine side chain is in a local charged environment on the surface of the protein, charge interactions with the acid form of the reagent may cause complications, and thus iodoacetamide may be a better choice. Modification using iodoacetic acid yields *S*-carboxymethyl cysteine, whereas iodoacetamide yields *S*-carboxamidomethyl cysteine. Both of these products are relatively stable in protein sequencing. The carboxymethyl group imparts a negative charge but the carboxamidomethyl group does not. Depending on the cysteine content of the protein of interest, the negative charge may alter the characteristics of the protein. Upon acid hydrolysis, *S*-carboxamidomethyl cysteine deamidates to *S*-carboxymethyl cysteine; this derivative is not as stable as some others to hydrolysis.

Alkylation with IAEDANS introduces a fluorescent label that allows sensitive fluorometric detection following chromatography, SDS-PAGE (*UNIT 10.1*), or electroblotting from gels (*UNIT 10.7*).

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N-ethylmaleimide (NEM) is a reagent containing a reactive double bond that has historically been used to modify cysteine residues and is still mentioned frequently in the literature. The advantages of NEM are that it is membrane permeable, it can be obtained in radioactive form, and the ethyl group can be replaced with other substituents, such as fluorescent adducts, to permit the introduction of reporter groups. Its disadvantages are that above pH 7 it is unstable and seems to have a higher propensity to react with amino groups and histidine. For these reasons, it is not recommended for routine use. If it is desirable for a particular application, however, NEM can be used in this protocol, but the reaction pH should be adjusted to 7.0. The adduct breaks down to *S*-succinylcysteine and ethylamine during acid hydrolysis.

This protocol can be used to prepare material suitable for amino acid analysis, sequence analysis, and proteolytic digestion (*UNITS 11.1 & 11.4*).

Materials

Protein, lyophilized
Tris/guanidine buffer, pH 8.0 (see recipe) or 0.1 M Tris·Cl, pH 8.0
50 mM dithiothreitol (DTT; 7.7 mg/ml)
Nitrogen
Alkylating agent (see recipe)
2-mercaptoethanol (2-ME; 14.4 M)
0.5- to 2.5-ml microcentrifuge tube

Additional reagents and equipment for desalting modified protein (see Support Protocol 3)

1. Dissolve the protein in a minimum volume of Tris/guanidine buffer to give a protein concentration ≥100 µg/ml in a volume between 50 and 500 µl, if possible.

   Buffering capacity and pH are critical, so interference by other components in the protein sample must be considered and removed, if necessary, prior to modification. Guanidine·HCl may be omitted from the buffers (i.e., 0.1 M Tris·Cl may be used) if protein denaturation is not desired.

   Use Tris/guanidine buffer, pH 7.0, or 0.1 M Tris·Cl, pH 7.0, if NEM is used as an alkylating agent.

2. Add DTT to give a 10-fold molar excess over expected disulfides.

   To calculate the amount of DTT required, assume all cysteines are present as disulfides and use the following equation:

   (µg protein) (nmol protein/µg protein) (nmol disulfide/nmol protein) = nmol protein disulfide

   For example, for 500 µg of a 25,000-Da protein containing 8 cysteines the amount of disulfide is (500 µg protein) (1 nmol protein/25 µg) (4 nmol disulfide/nmol protein) = 80 nmol protein disulfide. A 10-fold molar excess of DTT requires

   10 (nmol protein disulfide) = nmol DTT

   For the 25-kDa protein, this is 10 (80 nmol protein disulfide) = 800 nmol DTT. To calculate the amount of DTT solution required use the following equation:

   (nmol DTT) (1 µl/nmol DTT in stock solution) = µl DTT

   For a 50 mM DTT stock solution, this becomes (800 nmol DTT) (1 µl/50 nmol DTT) = 16 µl of 50 mM DTT.

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3. Flush nitrogen over the surface of the liquid and seal the microcentrifuge tube. Incubate 1 hr at 37°C.

4. Add alkylating agent to give a 5-fold molar excess of reagent over total thiols in solution.

   A 0.2 M stock solution of alkylating agent contains 200 nmol/µl.

   Total thiols in solution includes both protein thiol and reductant thiol. DTT contributes 2 moles of thiol per mole of reagent. To obtain a 5-fold molar excess, the amount of alklyating agent required is

   5 [(nmol DTT) (2 nmol SH/nmol DTT) + (nmol protein disulfide) (2 nmol SH/disulfide)] = nmol alkylating agent

   In the example here, the calculation is 5 [(800 nmol DTT) (2 nmol SH/nmol DTT) + (80 nmol protein disulfide) (2 nmol SH/disulfide)] = 8800 nmol. The volume of alkylating agent required is therefore

   (nmol alkylating agent required) (1 µl/*x* nmol alkylating agent in stock solution) = µl alkylating agent

   For this example, with alkylating agent at 200 nmol/µl, this is (8800 nmol) (1 µl/200 nmol) = 44 µl.

5. Flush nitrogen over the surface of the liquid and seal the microcentrifuge tube. Incubate 1 hr at 37°C in the dark.

   When iodinated reagents are used, exclusion of light is important to minimize the chance of formation of iodine from available iodide ions. As the reaction proceeds, it generates protons that will tend to make the solution more acidic if the buffer is not strong enough. If large amounts of protein (>5 mg) are being modified, it is advisable to monitor the pH and readjust to 8.0 as necessary.

6. Add ≥10-fold excess of 2-ME relative to the alkylating agent.

   A 10-fold excess of 2-ME requires 10 (8800 nmol alkylating agent) = 88 µmol 2-ME, which corresponds to (88 µmol) (1 µl/14.4 µmol) = 6 µl. As long as the 2-ME is in molar excess relative to the modifying reagent, the volume of 2-ME added can be rounded up to the nearest microliter or most convenient pipetting volume. For instance, if 1.3 µl is calculated, 2 to 5 µl can be added without harm.

7. Desalt the alkylated protein (see Support Protocol 3).

ALKYLATION OF A PROTEIN OF UNKNOWN SIZE AND COMPOSITION WITH HALOACYL REAGENTS OR *N*-ETHYLMALEIMIDE

ALTERNATE PROTOCOL 1

If the size and number of cysteines present in a protein are unknown follow procedure for a protein of known size and composition (see Basic Protocol 1), assuming that the protein is large (i.e., ~100,000 Da) and contains a high amount of cysteine (i.e., 50 cysteines = 25 disulfides per molecule) for the calculations.

ALKYLATION OF ≤50 µg PROTEIN WITH HALOACYL REAGENTS OR *N*-ETHYLMALEIMIDE

ALTERNATE PROTOCOL 2

Reduction and alkylation of small amounts of protein requires the use of very small volumes of reagents based on the calculations in Basic Protocol 1. If <50 µg protein is being alkylated, whether or not the composition and size are known, excess reagents can be used at each step. Follow procedure as for larger amounts (see Basic Protocol 4) but use 2 µl of 50 mM DTT, 6 µl of 0.2 M alkylating reagent, and 2 µl of 2-ME.

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ALKYLATION WITH 3-BROMOPROPYLAMINE

Use of 3-bromopropylamine is especially well suited to the identification of cysteine in automated amino-terminal protein sequencing methods (Jue and Hale, 1993). The modified cysteine, as aminopropylcysteine, is stable and well resolved in both amino acid analysis and protein sequencing separations (Jue and Hale, 1994). Furthermore, 3-bromopropylamine does not cause preview in protein sequencing, which is a potential problem with 4-vinylpyridine (see Basic Protocol 4). Preview in protein sequencing is the appearance of an amino acid residue in a sequencing cycle just prior to the cycle in which it would normally be cleaved from the protein.

Materials

Protein, lyophilized
20 mM and 2 M dithiothreitol (DTT) in 0.5 M Tris·Cl (pH 8.2)/6 M guanidine·HCl
0.5 M 3-bromopropylamine (see recipe)
Nitrogen
0.5- to 2.5-ml microcentrifuge tube

Additional reagents and equipment for desalting modified protein (see Support Protocol 3)

1. Dissolve protein in a minimum volume of 20 mM DTT/0.5 M Tris·Cl/6 M guanidine·HCl.

   Enough reducing power for ~200 nmol of –SH is provided by 100 µl of 20 mM DTT/Tris·Cl/guanidine·HCl. This amount of –SH is comparable to 1 mg of a 50,000-Da protein containing 10 cysteines. Alternatively, the necessary amount can be calculated as described in the haloacid/NEM procedure (see Basic Protocol 1, step 2). If more reducing power is required, either use more volume or increase the concentration of DTT in the buffer.

2. Flush with nitrogen over the surface of the liquid and seal the microcentrifuge tube. Incubate 1 hr at 60°C.

3. Add ⅕ vol of 0.5 M 3-bromopropylamine.

   For example, if the protein is dissolved in a total of 100 µl, add 20 µl of 3-bromopropylamine.

4. Flush with nitrogen over the surface of the liquid and seal the microcentrifuge tube. Incubate 2 hr at 60°C in the dark.

5. Add ⅕ vol of 2 M DTT/0.5 M Tris·Cl/6 M guanidine·HCl.

   For example, if the protein is in a total of 120 µl at this point, add 24 µl of 2 M DTT/Tris·Cl/guanidine·HCl.

6. Desalt the modified protein (see Support Protocol 3).

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ALKYLATION WITH *N*-(IODOETHYL)-TRIFLUOROACETAMIDE

Introduction of a primary amine by conversion of cysteine to *S*-(2-aminoethyl)-cysteine introduces a trypsin-sensitive cleavage site. Previously, this was accomplished using ethyleneimine, which is now restricted as a hazardous material. However, *N*-(iodoethyl)-trifluoroacetamide has been shown to be a comparable substitute (Schwartz et al., 1980); this reagent is available from Pierce Chemical as Aminoethyl-8 Reagent.

Materials

Protein, lyophilized
0.2 M *N*-ethylmorpholine acetate (pH 8.1)/6 M guanidine·HCl
50 mM dithiothreitol (DTT; 7.7 mg/ml)
Nitrogen
Aminoethyl-8 Reagent (Pierce Chemical)
Methanol
2 N acetic acid
0.5- to 2.5-ml microcentrifuge tube

Additional reagents and equipment for desalting modified protein (see Support Protocol 3)

1. Dissolve protein in a minimum volume of 0.2 M *N*-ethylmorpholine acetate (pH 8.1)/6 M guanidine·HCl.

2. Add DTT to a 10-fold molar excess over protein disulfide.

   Calculate the amount of 50 mM DTT required as described in the haloacid/NEM procedure (see Basic Protocol 1, step 2).

3. Flush with nitrogen and seal the microcentrifuge tube. Incubate 2 hr at room temperature.

4. Dissolve Aminoethyl-8 Reagent in a volume of methanol to give a 25-fold molar excess of reagent to total thiol groups and a final methanol concentration of 10% (v/v) relative to total reaction volume.

   To calculate the number of micrograms of reagent required use the following equation:

   25[(µg protein)(nmol protein/µg protein)(nmol disulfide/nmol protein)(2 nmol SH/nmol disulfide) + (µl DTT added)(nmol DTT/µl)(2 nmol SH/nmol DTT)](0.267 µg reagent/nmol) = µg Aminoethyl-8 Reagent

   For example, for 500 µg of a 25,000-Da protein containing 8 cysteines, the amount of reagent would be 25[(500 µg protein)(1 nmol protein/25 µg)(4 nmol disulfide/nmol protein)(2 nmol SH/nmol disulfide) + (16 µl DTT added)(50 nmol DTT/µl)(2 nmol SH/nmol DTT)](0.267 µg reagent/nmol) = 11,748 µg or 11.7 mg reagent.

   Dissolve the reagent in a volume of methanol equal to 0.11 × sample volume. If the volume of methanol required is too low to handle easily, increase the volume of the sample by adding 0.2 M N-ethylmorpholine acetate (pH 8.1)/6 M guanidine·HCl. Keep the final methanol concentration of the reaction at 10% (v/v).

   The solubility of Aminoethyl-8 Reagent in methanol is ~450 mg/ml.

5. Add one-half of the Aminoethyl-8 Reagent/methanol solution. Incubate 1 hr at room temperature.

6. Add the remaining Aminoethyl-8 Reagent. Continue the incubation an additional 1 to 1.5 hr at room temperature.

7. Acidify reaction to ~pH 5.5 with 2 N acetic acid.

8. Desalt modified protein (see Support Protocol 3).

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BASIC PROTOCOL 4

ALKYLATION WITH 4-VINYLPYRIDINE

Modification of cysteine with 4-vinylpyridine is simple and efficient. The resulting *S*-pyridylethyl cysteine derivative is stable to acid hydrolysis and also performs well in automated amino-terminal protein sequencing methods. However, there is evidence that some *N*-alkylation of the amino terminus of the protein may take place (Hempel et al., 1986). Upon reaction with phenylisothiocyanate (PITC), this adduct is unstable to the basic coupling conditions of the sequencing cycle, causing the alkylated amino acid to be released from the peptide and allowing the next amino acid to react with PITC. The result will be that the first cycle of sequencing can show some preview of the second residue and so on. The following protocol has been adapted from Applied Biosystems User Bulletin Number 28 (Hawke and Yuan, 1987).

Materials

1 M Tris·Cl (pH 8.5)/4 mM EDTA
8 M guanidine·HCl
Protein, lyophilized
10% (v/v) 2-mercaptoethanol (2-ME)
Argon
4-vinylpyridine, fresh
0.5- to 2.5-ml microcentrifuge tube

Additional reagents and equipment for desalting modified protein (see Support Protocol 3)

1. Mix 1 part 1 M Tris·Cl (pH 8.5)/4 mM EDTA to 3 parts 8 M guanidine·HCl to prepare the working buffer (0.25 M Tris·Cl/1 mM EDTA/6 M guanidine·HCl final).
2. Dissolve protein in <50 µl working buffer to a final concentration of 0.1 to 10 µg/µl and add 2.5 µl of 10% 2-ME.

   The procedure presented in this protocol will accommodate ≤500 µg of protein, but it can be scaled up for larger amounts. The reaction volume should be kept to a minimum and the amounts of reductant, alkylating reagent, and quenching reagent should be increased proportionally to the amount of protein. For example, if 750 µg of protein is used, reagent volumes should be increased by 50%.

3. Flush with argon and seal the microcentrifuge tube. Incubate 2 hr at room temperature.
4. Add 2 µl undiluted 4-vinylpyridine and mix.

   Only colorless 4-vinylpyridine that has been stored under argon at –20°C should be used.

5. Flush microcentrifuge tube with argon and seal the container. Incubate 2 hr at room temperature in the dark.
6. Either quench the reaction with 2.5 µl of 10% 2-ME and desalt, or desalt immediately with a rapid technique such as reversed-phase HPLC or membrane adsorption using a ProSorb cartridge (see Support Protocol 3).

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ALKYLATION WITH ACRYLAMIDE

BASIC PROTOCOL 5

Although the use of acrylamide to alkylate cysteine is not widespread, it is a viable alternative. The cysteine-*S*-β-propionamide derivative is stable to automated amino-terminal sequencing and yields a readily identifiable phenylthiohydantoin (PTH)-cysteine-*S*-β-propionamide. Use of acrylamide (Brune, 1992) may circumvent some problems associated with the use of 4-vinylpyridine (see Basic Protocol 4 and Commentary).

Materials

Protein, lyophilized
0.3 M Tris·Cl, pH 8.3
50 mM dithiothreitol (DTT; 7.7 mg/ml; optional)
6 M acrylamide
10% (v/v) 2-mercaptoethanol (2-ME)
0.5- to 1.5-ml microcentrifuge tube

Additional reagents and equipment for desalting modified protein (see Support Protocol 3)

CAUTION: Acrylamide monomer is neurotoxic. A mask should be worn when weighing acrylamide powder. Gloves should be worn while handling the solution, and the solution should not be pipetted by mouth.

1. In a microcentrifuge tube, dissolve protein in a minimal volume (<100 µl, if possible) of 0.3 M Tris·Cl, pH 8.3, to give a protein concentration ≥100 µg/ml.
2. If reduction is desired, add DTT to yield a 10-fold molar excess relative to expected disulfides (see Basic Protocol 1, step 2).
3. Add acrylamide to a final concentration of 2 M. Incubate 2 hr at 37°C.

   Alternatively, 9.7 M dimethylacrylamide (available as a premade solution from Aldrich) can be added to a final concentration of 2 M. Dimethylacrylamide produces a more hydrophobic derivative that in turn produces a later-eluting adduct of PTH-Cys. Acrylamide and dimethylacrylamide can be used interchangeably; the choice is a matter of personal preference (Brune, 1992).
4. Either quench the reaction with 2.5 µl of 10% 2-ME and desalt, or desalt immediately with a rapid technique such as reversed-phase HPLC or membrane adsorption using a ProSorb cartridge (see Support Protocol 3).

SULFITOLYSIS

BASIC PROTOCOL 6

Sulfitolysis is a mild procedure for cleavage of protein disulfide bonds, and the resultant *S*-sulfocysteine is easily reduced back to native cysteine with sulfhydryl compounds, such as 2-mercaptoethanol (2-ME; Chan, 1968). Only disulfides are reactive, but cysteine in proteins can be modified by including catalytic levels of free cysteine (or other sulfhydryl compound), which will drive the reaction through formation of mixed disulfides. The *S*-sulfocysteine derivative is stable only at neutral or acidic pH. For these reasons, this procedure is useful only in a limited number of applications; it is suitable when subsequent removal of the modifying group is an issue.

Materials

Protein, lyophilized
Sodium sulfite reagent buffer (see recipe)
4 M urea/1 M Tris·Cl, pH 7.5
2-mercaptoethanol (2-ME)
0.5- to 2.5-ml microcentrifuge tube

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Additional reagents and equipment for desalting modified protein (see Support Protocol 3)

Cleave disulfide bonds

1. Dissolve protein in sodium sulfite reagent buffer to a final concentration of 2 mg/ml. Incubate 1 hr at 25°C in an open microcentrifuge tube.
2. Desalt modified protein (see Support Protocol 3).

Reverse the modification

3. Dissolve the *S*-sulfocysteine protein in 4 M urea/1 M Tris·Cl, pH 7.5, to give a concentration of ~1 mg/ml.
4. Add 2-ME to a final concentration of 0.7 M. Incubate 2 hr at 25°C.
5. Desalt the protein (see Support Protocol 3).

BASIC PROTOCOL 7

OXIDATION WITH PERFORMIC ACID

Modification of cysteine by performic acid oxidation is most commonly used in conjunction with amino acid compositional analysis to produce a derivative of cysteine (cysteic acid) that is stable to hydrolysis. The procedure is not recommended for modification of cysteine for purposes other than quantitation of cysteine by amino acid analysis. Conversion of cysteine to cysteic acid with this protocol is simple and quantitative. However, performic acid is a very strong oxidizer and several other amino acids such as histidine, tyrosine, tryptophan, methionine, serine, and threonine are adversely affected (Hirs, 1967). These other affected amino acids must therefore be quantitated by amino acid analysis of a separate aliquot. Analysis of the performic acid–treated sample can be normalized to the untreated sample by comparing the levels of stable amino acids such as aspartic acid (Asp + Asn) and glutamic acid (Glu + Gln). The following protocol is intended for samples to be used for amino acid analysis.

CAUTION: This entire procedure should be performed in a fume hood.

Materials

Formic acid
30% hydrogen peroxide
Protein, lyophilized and chilled
48% (v/v) hydrobromic acid
Ice bath

1. Prepare fresh performic acid by adding 1 ml of 30% hydrogen peroxide to 9 ml formic acid. Mix and incubate 2 hr at room temperature.
2. Chill performic acid in an ice bath to 0°C.
3. To a chilled tube containing lyophilized protein, add sufficient chilled performic acid to thoroughly dissolve the protein. Incubate 1 hr on ice.
4. Add 0.2 ml hydrobromic acid.
5. Dry sample by lyophilization or Speedvac evaporation.

The sample is now ready for hydrolysis for amino acid analysis.

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AIR OXIDATION TO A DISULFIDE

BASIC PROTOCOL 8

Successful formation of the correct disulfide bonds in a protein or peptide is a critical step in achieving a biologically active product from chemical peptide synthesis or protein expression systems. The protocol presented here is very simplistic and the complexities of correct disulfide formation in proteins may require empirically derived combinations of denaturants and reducing agents (Noiva, 1994; Chau and Nelson, 1992; Zhang and Snyder, 1991). This protocol works best for small peptides such as those obtained from peptide synthesis procedures.

Materials

Protein or peptide, lyophilized
0.1 M ammonium bicarbonate

Additional reagents and equipment for reversed-phase HPLC (*UNIT 11.6*) and desalting oxidized protein (see Support Protocol 3)

1. Dissolve protein or peptide in 0.1 M ammonium bicarbonate.

   To reduce the likelihood of intermolecular disulfide formation, keep the protein or peptide concentration <0.25 to 0.5 mg/ml.

2. Gently stir several days with vessel open to air.

3. Monitor effects of oxidation by changes in elution position on reversed-phase HPLC relative to fully reduced starting product (more sensitive for smaller peptides).

   Alternatively, monitor the decrease in free sulfhydryl content colorimetrically with Ellman Reagent (see Support Protocol 2).

4. Desalt the oxidized protein (see Support Protocol 3).

ALKYLATION OF CYSTEINE IN PROTEIN APPLIED TO SEQUENCER MEMBRANES

BASIC PROTOCOL 9

Modification of cysteine in samples applied directly to sequencer membranes can be employed as an alternative to modification in solution. This procedure minimizes sample loss by reducing the manipulation required for derivatization and sample cleanup. Although this procedure has been found to be generally applicable, there are instances where it has failed with certain proteins, for reasons that are not clear. If a negative result is obtained, alkylation in solution followed by application to a polyvinylidene difluoride (PVDF) membrane is a reasonable alternative (see Support Protocol 3).

Tributylphosphine is used as a reductant in this procedure. Although its use is not widespread, this compound can be useful in cleavage of disulfide bonds for several reasons. First, only a slight excess (5% to 20%) over protein disulfide groups is necessary. Second, subsequent alkylation can take place in the presence of tributylphosphine without generating additional reaction by-products and with no allowance for thiols other than those associated with the protein. Finally, tributylphosphine is soluble in organic solvents, which makes it well suited to concomitant in situ reduction and alkylation immediately prior to protein microsequencing.

The following protocol is adapted from a Perkin Elmer Applied Biosystems Division (PE-ABD) User Bulletin to prepare samples for sequencing with a PE-ABD sequencer (Hawke and Yuan, 1987). It can be modified for use with other sequencers by setting up a wash cycle similar to that used on the PE-ABD sequencer (described in step 4).

CAUTION: Tributylphosphine is a hazardous reagent; proper precautions should be used when handling it.

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Materials

Protein
Reduction/alkylation cocktail (see recipe)
Sequencer reagents (Perkin-Elmer or equivalent)

Biobrene coated glass fiber disk, precycled
PE-ABD sequencer, cartridge (Perkin-Elmer) or equivalent

1. Apply protein to a precycled Biobrene coated glass fiber disk in the usual manner for sequencing and dry.
2. Pipet 20 µl reduction/alkylation cocktail onto the glass fiber disk.
3. Immediately reassemble the cartridge.
4. Execute the 4-VP cycle:

   Deliver coupling buffer for 60 sec
   Pause 50 min
   Dry with argon 5 min
   Wash 5 min with butyl chloride
   Dry with argon 2 min
   Deliver coupling buffer for 1 min
   Dry with argon 2 min
   Wash with ethyl acetate for 5 min
   Dry with argon 2 min.

   The reaction takes place during the 50-min pause.

5. Start sequencing run.

SUPPORT PROTOCOL 1

RECRYSTALLIZATION OF IODOACETIC ACID

Good quality iodoacetic acid (IAA) should be available from chemical companies such as Aldrich; IAA crystals should be white with no yellow tint. If the IAA has yellowed during storage, its use can result in iodination of the protein. The quality of the IAA can be significantly improved by recrystallizing it from petroleum ether until the yellow color is gone.

CAUTION: Ether is very flammable. Use it in a fume hood away from flame.

Materials

Iodoacetic acid (IAA)
Diethyl ether
Petroleum ether, ice cold

Buchner funnel
Whatman No. 1 filter paper or equivalent
Amber bottle with Teflon-lined lid

1. Dissolve 10 to 15 mg IAA in a minimal volume of diethyl ether (4 to 5 ml). Stir until the IAA dissolves.
2. Pour into a beaker containing 350 ml cold petroleum ether.
3. Collect crystals on a Buchner funnel with Whatman No. 1 filter paper.

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4. Dry 24 hr under vacuum.

   If crystals are not snow-white, repeat steps 1 to 4 until no yellow tint remains.

5. Store crystals in a tightly sealed amber bottle with a Teflon-lined lid at –20°C.

COLORIMETRIC QUANTITATION OF FREE SULFHYDRYLS

SUPPORT PROTOCOL 2

Free sulfhydryl content can be quantitatively determined with Ellman reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid) from Pierce Chemical. Molar extinction coefficients of thionitrobenzoate, the colored species generated when the reagent reacts with a free thiol, are given for various solutions in Table 15.1.2.

The sensitivity of the reaction is in the low nmol/ml range for sulfhydryl groups. This method is often not sensitive enough for the amounts of protein available when proteins from natural sources are being analyzed, but it is well-suited to synthetic peptides or recombinant proteins that are usually available in larger quantities.

Materials

0.1 M sodium phosphate, pH 8.0 (*APPENDIX 2E*)
Reagent solution: 4 mg/ml Ellman reagent/0.1 M sodium phosphate, pH 8.0
Cysteine standard stock solution (see recipe)
Unknown sample
13 × 100-mm clear test tubes

1. Prepare a cysteine standard curve by adding different amounts of cysteine standard stock solution to separate tubes—e.g., 25 µl, 50 µl, 100 µl, 150 µl, 200 µl, and 250 µl. Add ≤250 µl of each unknown to separate tubes. Bring the volume in each tube to a total of 250 µl with 0.1 M sodium phosphate, pH 8.0. Add 250 µl 0.1 M sodium phosphate, pH 8.0, to a blank tube.

   The cysteine content of an unknown should fall within the range of the standard curve (37.5 to 375 nmol). If a rough estimate of the cysteine content of an unknown cannot be made, at least two different amounts—e.g., 25 µl and 250 µl—should be tested.

2. Add 50 µl reagent solution and 2.5 ml of 0.1 M sodium phosphate, pH 8.0, to each tube. Mix and incubate 15 min at room temperature.
3. Measure absorbance at 412 nm.
4. Plot the A_{412} values obtained for the standards after subtracting the A_{412} value for the blank to derive a standard curve.
5. Use this curve to determine the free sulfhydryl content for the experimental samples.

Table 15.1.2 Molar Extinction Coefficients of Thionitrobenzoate in Various Solutions[a]

Solvent	ε_{412}
2% SDS	12,500
0.1 M phosphate (pH 7.27)/1 mM EDTA	14,150
Buffered 6 M guanidine·HCl	13,700
6 M guanidine·HCl	13,880
8 M urea	14,290

[a]Taken from the Pierce Chemical technical bulletin for product number 22582.

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SUPPORT PROTOCOL 3

DESALTING THE SAMPLE AFTER CYSTEINE MODIFICATION

Except for the in situ method (Basic Protocol 9), all of the protocols presented leave the protein in the presence of high salt and reaction by-products which are usually not compatible with subsequent analytical techniques. This problem may be remedied by one of the following desalting methods. The methods are presented in increasing order of technological complexity. Protein recovery is a concern because small quantities of protein may be lost in dialysis or gel filtration. Modern microanalytical techniques typically work with <100 pmol protein, which can easily disappear due to nonspecific binding to dialysis tubing, gel-filtration columns, and ultrafiltration devices. The most commonly applied desalting techniques currently used for picomole quantities of proteins are ProSpin and ProSorb cartridges (Perkin-Elmer) for direct sequence analysis, or reversed-phase HPLC (*UNIT 11.6*) using narrow or microbore columns.

Dialysis. Dialysis is well suited to large-scale (milligram quantity) desalting needs. The protein is simply placed in dialysis tubing of an appropriate molecular weight cut-off (MWCO) such that the protein will be retained. The tubing is placed in a large vessel of appropriate buffer and gently stirred. After two or three changes of buffer over 24 to 48 hr, the composition of buffer in the tubing is now effectively the same as in the large vessel, due to the exchange of low-molecular-weight salts and other small molecules through the dialysis tubing. The volume inside the tubing will increase, so it is necessary to allow for this by leaving additional space in the dialysis tubing when the protein sample is added. Dialysis may be followed by lyophilization or Speedvac evaporation to concentrate the sample.

Some brands of dialysis tubing require special preparation before use. For more information on dialysis, see *UNIT 4.4 & APPENDIX 3B*.

Gel filtration (size-exclusion chromatography). Like dialysis, this method is better suited to large-scale desalting and is relatively inexpensive. It can be performed as a low-pressure bench-top technique using media such as Sephadex G-15 or G-25 resins or as a high-pressure liquid chromatography application (in which case it can be more readily done on a microscale). As with dialysis, buffer exchange may be accomplished with gel filtration, but subsequent concentration of the sample may be necessary. See *UNIT 4.4* and *UNIT 8.3* for additional details.

Centrifugal filtration. Centrifugal filtration with membrane cartridges (Amicon; *UNIT 4.4*) combines the buffer exchange of dialysis with a concentration step by using centrifugation to force liquid through a membrane of appropriate MWCO. The protein is retained, may be repeatedly washed, and is ultimately recovered in a very small volume. When routinely desalting the same protein, a Centricon cartridge can be reused; this can potentially increase recovery of protein as nonspecific binding sites become saturated.

Adsorption to a membrane. This method is well suited to samples that will subsequently be microsequenced because the resulting protein-containing polyvinylidene difluoride (PVDF) membrane can be placed directly in the reaction cartridge of a protein sequencer. ProSpin and ProSorb cartridges (Perkin-Elmer) are useful for this procedure. ProSpin uses centrifugation to force liquid through the PVDF membrane. ProSorb, a more recent development that has superseded ProSpin, uses an absorptive process to transfer protein to the membrane. Both cartridges require a homogeneous protein solution because the PVDF membrane will bind all protein present. Refer to manufacturer's instructions for procedures with ProSpin and ProSorb cartridges.

Reversed-phase HPLC (RP-HPLC). This method is the most technologically complex, because it requires the use of a complete high-pressure liquid chromatography (HPLC)

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system. For microscale work, the HPLC system should be configured for narrow-bore (2-mm) or micro-bore (1-mm) work because larger column diameters are better suited to milligram quantities of protein. The most common elution method uses a two-component system with 0.1% (v/v) trifluoroacetic acid in water (buffer A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (buffer B). Protein is detected by ultraviolet absorbance at 210 or 280 nm. The protein is acidified and injected onto a column that has been equilibrated with 100% buffer A, then eluted either isocratically with 80% buffer B or with an increasing gradient of buffer B. The column eluant is monitored and the appropriate fraction(s) are pooled, if necessary, and exposed to a jet of nitrogen or argon gas to evaporate the majority of the trifluoroacetic acid and acetonitrile phase. The remaining, predominantly aqueous, phase is lyophilized or vacuum concentrated. When working at narrow or microbore scale, the recovered fraction volumes are small enough (<100 µl) that they may be directly loaded onto a protein sequencer sample support. Ideally desalted samples should be lyophilized and stored in a freezer in the presence of a dessicant. Alternatively, they can be stored frozen in the dissolved state.

REAGENTS AND SOLUTIONS

Use Milli-Q water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Alkylating agent

Prepare buffer for alkylating agent by adding 1 g guanidine·HCl to 1 ml 0.95 M Tris·Cl, pH 8.0 (0.5 M Tris·Cl and 6 M guanidine·HCl final). To this buffer add one of the following alkylating agents to a final concentration of 0.2 M:

Iodoacetic acid (FW 186)
Iodoacetamide (FW 185)
N-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS, FW 434)
N-ethylmaleimide (FW 125)

A concentration of 0.2 M provides 200 nmol alkylating agent/µl.

If N-ethylmaleimide is used as the alkylating agent, prepare the buffer with 0.95 M Tris·Cl, pH 7.0.

3-Bromopropylamine, 0.5 M

0.5 M 3-bromopropylamine
0.5 M Tris·Cl, pH 8.2
6 M guanidine·HCl
Prepare fresh

Cysteine standard stock solution, 1.5 mM

Dissolve 26.34 mg cysteine hydrochloride monohydrate in 100 ml 0.1 M sodium phosphate, pH 8.0, immediately before use.

Reduction/alkylation cocktail

2 µl tri-*n*-butylphosphine
5 µl 4-vinylpyridine
100 µl acetonitrile
Prepare fresh

Sodium sulfite reagent buffer

0.05 M sodium sulfite
0.2 mM cysteine
8 M urea *or* 6 M guanidine·HCl
0.1 M Tris·Cl, pH 8.4
Prepare fresh

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Tris/guanidine buffer

1 g guanidine·HCl (6 M final)
1 ml 0.19 M Tris·Cl, pH 8.0 (0.1 M final)
Store at 25°C

For N-ethylmaleimide, use Tris·Cl, pH 7.0.

COMMENTARY

Background Information

Cysteine is the most reactive residue commonly found in proteins and peptides. The thiol group, in its unprotonated state, is strongly nucleophilic and reacts with a wide variety of reagents. In addition to the free thiol, cysteine also occurs in disulfide linkage where it is relatively unreactive.

The methods most commonly used to modify cysteines involve alkylating reagents, including active halogen compounds (Basic Protocols 1, 2, and 3) and compounds containing reactive double bonds (Basic Protocols 1, 4, 5, and 9). Cysteine thiols can also be oxidized either with stong oxidizing agents such as performic acid (resulting in conversion of the thiol to a sulfonic acid group; Basic Protocol 7) or with weak oxidizers such as oxygen (resulting in formation of disulfides; Basic Protocol 8; see Fig. 15.1.1). Finally, cysteine residues can form mixed disulfides with other thiol reagents. Although the formation of mixed disulfides has been used to modify protein thiols (Wynn and Richards, 1995) and to provide stabilizing adducts in amino acid analysis (Barkholt and Jensen, 1989; Hoogerheide and Campbell, 1992), these methods are omitted here, for several reasons. Unlike alkylations, which are irreversible, mixed disulfide formation is freely reversible and the disulfides may thus be lost during subsequent manipulations. In addition, unless subsequent conditions are carefully controlled, disulfide scrambling may occur once the excess disulfide reagent is removed. Moreover, many of the reagents that have been reported in the literature for this purpose are not commercially available and must be synthesized prior to use. Thus, modification by mixed disulfide formation does not usually lend itself well to routine modification. However, the procedures for use of mixed disulfides in amino acid analysis (Barkholt and Jensen, 1989; Hoogerheide and Campbell, 1992) use commercially available reagents and appear to be quite accurate.

Critical Parameters and Troubleshooting

With few exceptions, disulfides generally need to be reduced to free thiols before they

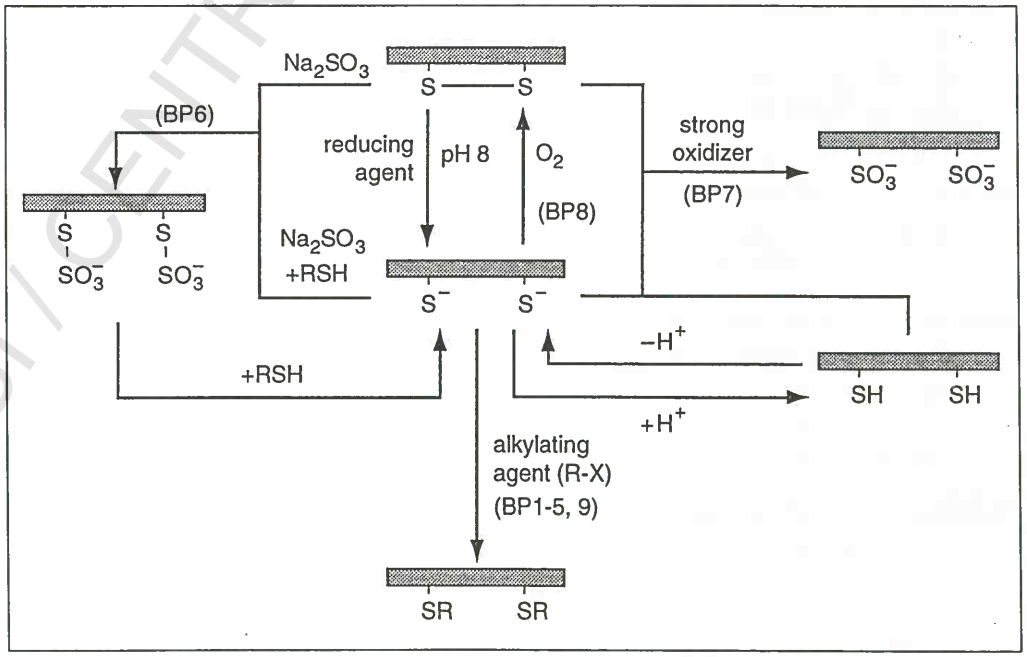

Figure 15.1.1 Modification of cysteine. Interrelationship of the cysteine-modifying reactions described in this unit. Abbreviations: BP, Basic Protocol; R, alkyl group.

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can be derivatized. In addition to cysteine, proteins and peptides contain other reactive groups that, under the right conditions, can also react with many of the reagents used to derivatize cysteine. These groups include lysine ε-amino groups and the imidazole nitrogens of histidine, both of which are nucleophilic in the unprotonated state, and methionine, which is a pH-independent nucleophile. However, derivatization of cysteine can be made highly specific because of its pK and high reactivity. For instance, at pH 8, the thiol group, which has a pK of 8.3, has a significant population of unprotonated and thus nucleophilic species, but other potential nucleophiles are either still largely protonated (lysine ε-amino group) or react so slowly compared to the thiolate anion that they remain essentially unreacted in the time it takes for complete modification of cysteine (histidine and methionine). Thus, pH and duration of the reaction are critical factors in performing cysteine modifications. If residues other than cysteine become alkylated, repeat the experiment reducing the time of alkylation and check that the pH is correct and stable during the alkylation reaction.

Any procedure that utilizes sulfhydryl-containing compounds to reduce disulfides prior to modification must take these sulfhydryl reagents into account when an alkylating reagent is added. The added sulfhydryl-containing compounds will also react with the alkylating reagent; in cases where the alkylating reagent is not in sufficient excess, they will consume the reagent and make it unavailable for the intended cysteine modification, resulting in only partial modification. Thus, it is essential to ensure that the alkylation reagent is in excess of the total thiol content of the sample.

Once the alkylation reaction is completed, any excess alkylating reagent that remains unreacted should be destroyed before proceeding. Conditions can change significantly with subsequent sample manipulation, and this may allow remaining alkylating reagent to react in an unwanted manner with other components of the protein. Moreover, if halogen-containing reagents are used, halide ions are formed as a product of the alkylation reaction, and prolonged exposure to halide ions may result in halogenation of the protein.

Occasionally specific cysteine residues will be resistant to alkylation with particular reagents. This is more apt to occur if the protein is not denatured prior to alkylation. For example, if the local environment of a protein thiol is charged, iodoacetic acid may not be as effective as an uncharged reagent such as iodoacetamide. If certain cysteine residues seem to be resistant to alkylation and a denaturing agent is being used, employing a different alkylating reagent may be helpful.

Anticipated Results

Alkylation of cysteine residues is generally rapid and specific if the reaction conditions are controlled and care is taken to assure that correct ratios of reagents are employed. Thus, rapid and selective modification of cysteine residues in high yield (>90%) can generally be expected.

Time Consideratons

Allow ~30 to 60 min for buffer and reagent preparation. With the exception of air oxidation of cysteines to disulfides (Basic Protocol 8), each protocol should take 2 to 4 hr. Production of disulfides by air oxidation is a slow process and will take from 1 to 7 days for completion. Recrystallization of iodoacetic acid (Support Protocol 1) takes 2 days. Assaying free sulfhydryls with Ellman reagent can be completed in 3 to 4 hr. The time required to desalt the sample after modification (Support Protocol 3) depends on which procedure is used and may also depend on the volume of sample: dialysis requires 24 to 48 hr; gel filtration requires 6 to 24 hr; centrifugal filtration requires 2 to 8 hr; adsorption to a membrane requires 1 to 6 hr; and HPLC requires 1 to 3 hr.

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Modification of Amino Groups

This unit describes group-specific modifications of amino groups. These reactions remain valid tools for early-stage evaluation of structure-function relationships, but are now valued even more for their applications in the preparation of bioconjugates, affinity columns, biosensors, and tagged macromolecules. Despite this shift in emphasis, a practical acquaintance with the classical amino group modifications (see Table 15.2.1) remains the key to a valuable toolbox of protein biochemistry methods.

The amino groups of a polypeptide chain may include one N-terminal α-amino group, if it is not blocked, and any number of ε-amino groups of lysine. When the N-terminus is blocked by cell-mediated acetylation, cyclization of N-terminal Gln to pyroglutamate, or any other less common mechanism, it is inaccessible to chemical modification. Side-chain amino groups may also undergo post-translational modification, as in the ε-*N,N,N*-trimethylation of Lys-115 in calmodulin, but they are unmodified and available for chemical modification in the great majority of cases. With the exception of N-terminal Pro, the free amino groups of proteins are primary amines.

All of these groups have broadly similar reactivities, but there is a major difference in the typical pK_a values for α-amino groups (pK_a ~8) and ε-amino groups (pK_a ~10). Selective modification of the N-terminus by careful selection of the reaction pH is an interesting route to site-specific tagging, but the usual numerical preponderance of ε-amino groups partly offsets the higher relative reactivity of the α-amino group. For reasons unconnected with pK_a, amino-terminal residues of proteins also participate in several specialized reactions that are not available to lysine side chains (see Table 15.2.2).

The most important reactions of amino groups are acylations, which yield amide (protein-NH-CO-R) or, less importantly, sulfonamide (protein-NH-SO_2-R) products (where R indicates a group added to the protein by formation of a new bond). Other valuable modifications are based on nucleophilic substitution, nucleophilic addition, and reductive alkylation. In all cases, the amino group acts as a nucleophile that attacks an electrophilic center to acquire a modifying group of atoms.

In particular, the reactions of amino groups with succinimidyl esters (see Basic Protocol 1) and isothiocyanates (see Basic Protocol 2) are broadly useful for the stable coupling to proteins of groups with useful, non-native functional properties. These include biotin for detection or recovery, fluorescent groups for biophysics or cytochemistry, cross-linking reagents for making bioconjugates, or metal-chelators that permit proteins to be loaded with radioisotopes for medical imaging or antitumor therapy. These applications require accurate product characterization, which preferably is performed by mass spectrometry (see Support Protocol). Succinic or acetic anhydrides may be used to change the charge state of protein amino groups (see Basic Protocol 3); reductive alkylations leave their charge unaltered but convert primary amines to secondary or tertiary amines (see Basic Protocol 4).

STRATEGIC PLANNING

Cysteine is most often modified for analytical purposes (*UNIT 15.1*), because its unmodified form is not reliably detected in amino acid analysis or Edman sequencing. The native structure of the protein is expendable in these applications, and denaturants are commonly used to ensure that sulfhydryls (which may otherwise be buried) are exposed to the reagents.

Amino group modification has quite a different history. Because they are reactive and accessible, amino groups were and are often modified under native conditions to assess their contributions to protein function. Today, however, they are increasingly modified

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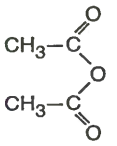
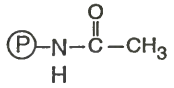
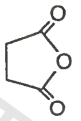
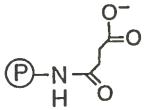
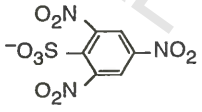
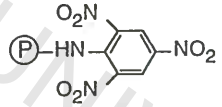
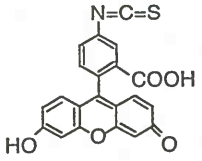
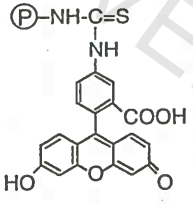
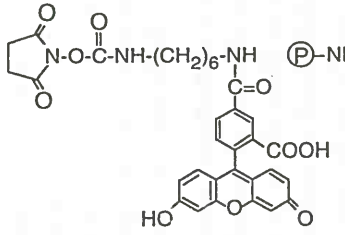
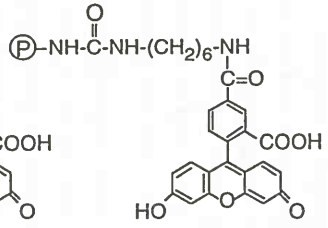
Table 15.2.1 Some Common Reagents for Modification of Amino Groups

Reagent	Application	Structure of reagent	Structure of modified amino group[a]	Reference(s)	Related reagents
Acetic anhydride	Acetylation; renders positively charged amino groups neutral			Means and Feeney (1971)	Acetylimidazole (reacts primarily with phenolic groups of tyrosine, but has significant side reaction with amino groups); iodoacetic anhydride (see Table 15.2.2)
Succinic anhydride	Succinylation; renders positively charged amino groups negative			Klotz (1967)	Maleic anhydride (Butler and Hartley, 1972); citraconic anhydride (Atassi and Habeeb, 1972); both are reversible by low pH, but modification with citraconic anhydride is more easily reversible
2,4,6-Trinitrobenzene-sulfonic acid (TNBS)	Arylation; adds a chromophore and can be used to measure free amino groups			Hirs (1967); Fields (1972)	2,4-Dinitrobenzenesulfonic acid (reacts very slowly); 2,4-dinitrofluorobenzene
Fluorescein isothiocyanate (FITC)	Addition by phenylthiocarbamylation of amino groups; couples a strongly fluorescent label to the protein			Banks and Paquette (1995)	Rhodamine isothiocyanate
Fluorescein succinimidyl ester	Alternative reagent for tagging with fluorescein; increasingly popular alternative to FITC			Banks and Paquette (1995)	5-Carboxytetramethylrhodamine succinimidyl ester

continued

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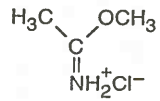
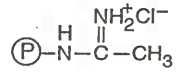
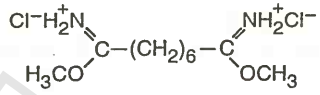
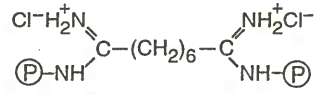
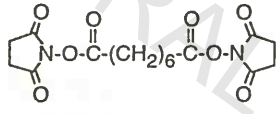
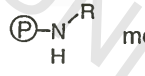
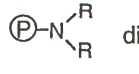
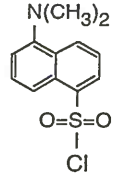
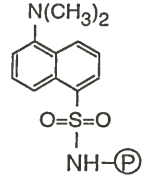
Table 15.2.1 Some Common Reagents for Modification of Amino Groups, continued

Reagent	Application	Structure of reagent	Structure of modified amino group[a]	Reference(s)	Related reagents
Methyl acetimidate hydrochloride	An imidoester; modifies amino groups to a strongly basic form			Inman et al. (1983)	
Dimethylsuberimidate dihydrochloride	Cross-linker; a homobifunctional imidoester			Tae (1983)	Related cross-linkers differ by length of alkyl chain, e.g. dimethyladipimidate; spacer chain may be thiol-cleavable, e.g., dimethyl-3,3'-dithiobispropionimidate
Disuccinimidyl suberate	Cross-linker; a homobifunctional succinimidyl ester				Bis-(sulfosuccinimidyl)suberate is a more water-soluble alternative
Aldehyde/ $NaBH_3CN$	Alkylates the amino group once or twice; amino group remains capable of being protonated	R-CHO $NaBH_3CN$	 monoalkylated  dialkylated	Jentoft and Dearborn (1983)	
Dimethylaminonaphthylsulfonyl chloride (dansyl chloride)	Reacts with an amino group to form a sulfonamide that is stable even to acid hydrolysis conditions				

[a]P, protein molecule.

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Table 15.2.2 Modification Reactions Specific for the Amino Terminus

Reagent	Application	Reagent	Structure of modified amino group	Reference	Comments
$NaIO_4$	Selectively oxidizes N-terminal Ser or Thr to an α-*N*-glyoxylyl-residue	IO_4^-	O=CH-CO-protein	Dixon and Fields (1972); Geoghegan and Stroh (1992); Alouani et al. (1995); Zhang and Tam (1996)	Creates a chemically unique site in the protein for conjugation with modifying group; adducts described include hydrazones, reduced hydrazones (alkyl hydrazides), oximes, and thiazolidines
Metal-catalyzed transamination	Converts the N-terminal residue to an α-oxo-acyl moiety	Metal [e.g., Ni(II)], Base (e.g., acetate), and amino acceptor (glyoxylate)	O=CR-CO-HN-protein[a]	Dixon and Fields (1972); Gaertner and Offord (1996)	Replaces the α-amino group with carbonyl oxygen; procedure considered prone to side reactions; when successful, can be used as the locus for further chemical modifications
Iodoacetic anhydride used at pH 6	Introduces a sulfhydryl-reactive iodoacetyl group at the amino terminus	$(I\text{-}CH_2\text{-}CO)_2O$	$I\text{-}CH_2\text{-}CO\text{-}HN$-protein	Wetzel et al. (1990)	Creates a chemically unique site in the protein for conjugation with sulfhydryl-containing modifying groups; has applications for cyclizing peptides

[a]R, side chain of the N-terminal residue.

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for preparative purposes, as they make convenient attachment points for the nonpeptide elements of molecular hybrids. Reactions done for either purpose are subject to the same issues at the planning stage. Key points to consider when preparing for such modifications are the reaction conditions, the extent of reaction desired, the method for adding reagents, and the technique for assessing results.

ε-Amino groups are largely protonated at pH <9, but it is their unprotonated form that is chemically reactive:

$$\underset{\text{protonated and unreactive}}{\text{protein-NH}_3^+} \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} \underset{\text{unprotonated and reactive}}{\text{protein-NH}_2}$$

Despite this, it is not necessary to set the reaction buffer at a very basic pH (e.g., pH 11) to ensure that all the amino groups in the protein are unprotonated. Doing so could be disastrous, in fact, because many modification reagents are labile to alkaline hydrolysis and proteins themselves undergo alkali-driven degradative reactions. It is only necessary to set and maintain a pH at which the amino groups are unprotonated *to an extent that permits a reasonable reaction rate*. This is usually pH 8 to 9 for reactions of protein amino groups with important reagents such as succinimidyl esters, sulfonyl halides, carboxylic acid anhydrides, and isothiocyanates. As unprotonated amino groups are consumed by the reaction, the original equilibrium distribution between protonated and unprotonated amino groups is continuously regained, so that all the amino groups can eventually be modified if desired. Elevated temperatures are hardly ever needed for the reactions considered here. A generous range of suitably reactive modifiers exists for amino groups, and most work can be done in the range of 0° to 22°C.

The extent of modification required is a function of the application. When coupling biotin to a protein using a sulfosuccinimidyl ester, the most desirable result might be the minimum extent of modification consistent with biotinylating a large fraction of the protein molecules. Other applications call for saturation of all amino groups with the modifying group. Whenever possible, a pilot experiment should be done in which small amounts of protein are exposed to a range of treatments with varied levels of modifier to assess the outcome.

Reagents must be added in a way that preserves their reactivity as well as respecting the sensitivity of the protein to elevated levels of organic solvent and pH. For example, ester reagents are base-labile and should be protected from exposure to alkaline conditions until they are added to the protein.

As to the means by which the result is evaluated, fast electrophoretic methods are excellent tools for this purpose; optical spectroscopy may give instant information regarding the introduction of a chromophore; and mass spectrometry (see Chapter 16) offers the greatest accuracy at the cost of more complexity and the need for expensive equipment.

AMIDATION USING A SUCCINIMIDYL ESTER

BASIC PROTOCOL 1

It is often useful to add a non-native functional property to a protein by modifying it with biotin, a fluorescent group, a metal chelator, or other selected group. The most versatile and reliable methods for this purpose use succinimidyl ester reagents. When water-solubility of the reagent needs to be enhanced or ensured, a sulfosuccinimidyl ester may be used instead. *N*-hydroxysuccinimide or *N*-hydroxysulfosuccinimide, each a good leaving group, is displaced from the carboxylic reagent by the attack of a protein amino group. The bond formed between the modifying reagent and the protein is a stable amide (see Fig. 15.2.1).

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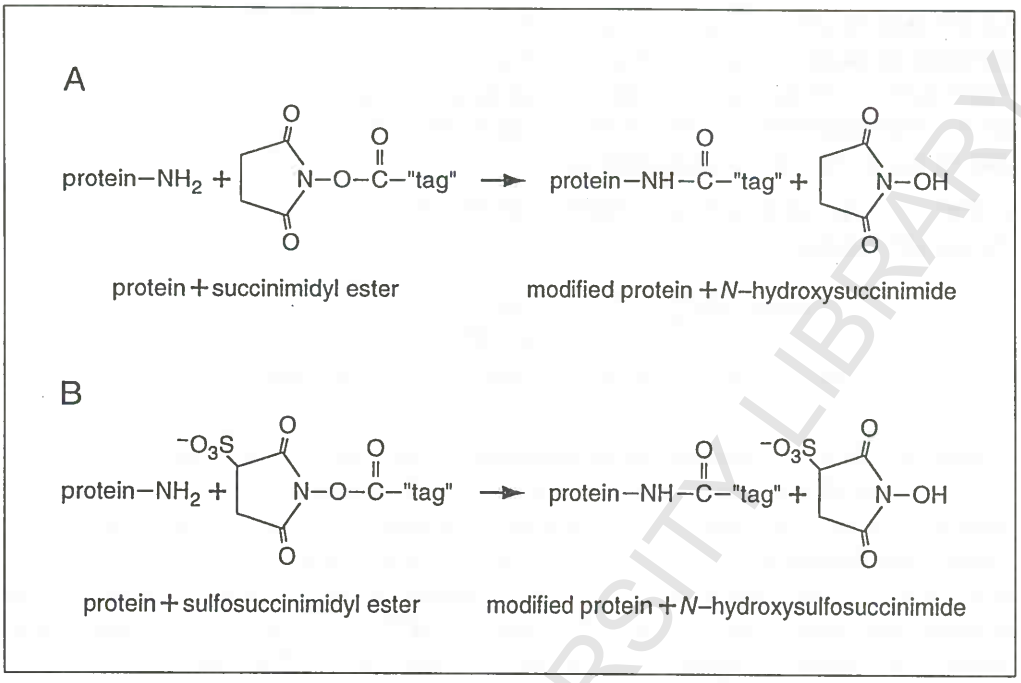

Figure 15.2.1 General representation of the modification of a protein amino group by (**A**) a succinimidyl ester and (**B**) a sulfosuccinimidyl ester. Sulfosuccinimidyl esters are more water-soluble than the succinimidyl esters of the same acids. The "tag" denoted in the figure represents the group added to the protein and may take many forms.

Numerous reagents from this group are commercially available from vendors such as Pierce and Molecular Probes (see *SUPPLIERS APPENDIX*), whose catalogs are useful resources; a concise review of these reagents was provided by Brinkley (1993). Biotin succinimidyl esters that include spacer arms of varying lengths are popular reagents for the production of biotinylated proteins; fluorescent reagents such as 5-carboxytetramethylrhodamine succinimidyl ester and fluorescein succinimidyl ester are convenient for conferring visible-range fluorescence on a protein. Some reactive media designed for protein immobilization are based on succinimidyl esters—e.g., Affi-Gel 10 (Bio-Rad) and HiTrap NHS Sepharose (Pharmacia Biotech).

Materials

0.1 to 10 mM succinimidyl ester reagent in acetonitrile, dimethylformamide (DMF), or dimethylsulfoxide (DMSO); *or* 0.1 to 10 mM sulfosuccinimidyl ester reagent in H_2O

Protein solution: 0.1 to 2 mM protein in 0.1 M sodium bicarbonate, pH not adjusted

Additional reagents and equipment for gel-filtration chromatography (*UNIT 8.3*) or dialysis (*UNIT 4.4* and *APPENDIX 3B*) and for mass spectrometry (see Chapter 16)

1. Add succinimidyl ester or sulfosuccinimidyl ester reagent to the protein solution ensuring a ≥2:1 molar excess of succinimidyl ester over the number of amino groups to be modified. Incubate ≥1 hr at room temperature.

   If the reagent has been dissolved in an organic solvent, before preparing the reaction mixture, use a small amount of the protein solution to test that the protein is soluble in the partly organic reaction mixture.

   If made up in acetonitrile, DMF, or DMSO, these reagents are stable; if made up in water, they will be more stable than if made up in the basic reaction buffer.

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2. Remove excess reagent and by-products from the protein by gel-filtration chromatography (*UNIT 8.3*), dialysis (*UNIT 4.4* and *APPENDIX 3B*), or HPLC.

   See UNIT 15.1 for additional advice on a number of these methods.

   Microconcentrators (e.g., Centricon, Amicon) are useful for rapidly reducing sample volumes and exchanging buffer; however, these devices may need to be treated with polyethylene glycol to minimize protein adsorption (see manufacturer's instructions).

3. Evaluate the extent of modification by mass spectrometry (see Chapter 16), UV-visible absorption spectroscopy (if the modifying group is chromophoric), or another suitable method as dictated by the nature of the modifying group.

ADDITION OF FLUORESCEIN ISOTHIOCYANATE

BASIC PROTOCOL 2

Fluorescence-activated cell sorting is a central technique of cell biology and frequently requires the use of antibodies that carry a fluorescent tag. Fluorescein isothiocyanate (FITC) is the reagent most commonly used to provide antibodies with the requisite fluorescence. The isothiocyanate functional group (–N=C=S, in which the carbon atom is the target for nucleophilic attack) readily reacts with amino groups at pH 9.0 to form phenylthiocarbamyl adducts that are stable at pH 7.0 (see Figure 15.2.2).

The following procedure is based on recommendations supplied by Molecular Probes for use with amine-reactive probes in general.

Materials

Protein, lyophilized or as a 5 to 20 mg/ml solution in 0.1 M sodium bicarbonate, pH 9.0
0.1 M sodium bicarbonate, pH 9.0 (pH adjusted with sodium hydroxide)
Fluorescein isothiocyanate (FITC)
Dimethylformamide (DMF) or dimethylsulfoxide (DMSO)
1.5 M hydroxylamine·HCl, pH 8.5, prepared fresh
Gel-filtration column containing chromatography medium (e.g., PD-10 column containing Sephadex G-25; Pharmacia Biotech), equilibrated in PBS (*APPENDIX 2E*)

1. If necessary, dilute or dissolve protein to 5 to 20 mg/ml in 0.1 M sodium bicarbonate, pH 9.0.
2. Dissolve FITC at 10 mg/ml in DMF or DMSO, sonicating or vortexing briefly if necessary.

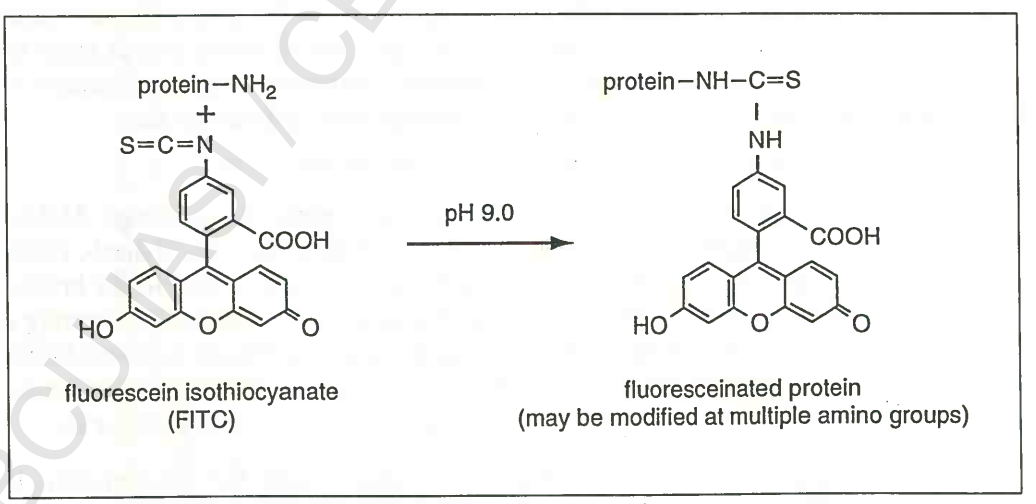

Figure 15.2.2 Modification of a protein amino group with fluorescein isothiocyanate.

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3. Slowly add 50 to 100 µl FITC per milliliter of protein solution while gently shaking the tube to mix. Incubate 1 hr at room temperature.

4. Stop the reaction by adding 0.1 ml of 1.5 M hydroxylamine·HCl, pH 8.5, per milliliter of reaction mixture.

   This provides excess amino groups to quench residual FITC.

5. Pass the reaction mixture over a gel-filtration column equilibrated with PBS.

   Dye-conjugated protein should emerge before all low-mass components, and should be stored at –70°C or used immediately.

BASIC PROTOCOL 3

SUCCINYLATION

At acidic, neutral, or mildly basic pH (<pH 8.5), typical protein amino groups are protonated and carry a positive charge. Succinic anhydride reacts with amino groups to give a stable modified derivative, which has negative charge at all pH values where its carboxylic group is not protonated, i.e., at values of pH >5. This modification can radically alter the solubility of a protein or its ability to participate in intermolecular associations. Klotz (1967) provides a practical guide to using succinic anhydride and related reagents (e.g., *S*-acetylmercaptosuccinic anhydride).

The protein is dissolved in a buffer of pH 8.0 to 9.0 that contains no potentially reactive amine. Tris must not be used, because it is a primary amine that will compete with protein amino groups for the anhydride. Buffers made with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), borate, or bicarbonate (see Table 15.2.3) are better choices. Succinic anhydride may be added as a solid, and its reactions with the protein take place in competition with its hydrolysis to succinic acid.

The reaction with amino groups (see Fig. 15.2.3) predominates, but tyrosine side chains may also be modified at the phenolic oxygen to a derivative that is stable at pH <5, and therefore may be present if the product of the modification reaction is examined directly by HPLC performed with solvents containing 0.1% trifluoroacetic acid. If this occurs or is feared to have occurred, the protein may then be treated with 1 M hydroxylamine·HCl, pH 7.0, ≥1 hr at 20°C to remove the modifying group from tyrosine. Amino groups remain modified under these conditions. Side reactions of succinic acid also occur with other amino acid side chains (Klotz, 1967), but these are so readily reversible that they are of little general importance.

Other types of modifications chemically related to succinylation are also important. This protocol may be used with acetic anhydride, a liquid reagent, in essentially the same fashion as with succinic anhydride. In general, succinylation of amino groups tends to promote the aqueous solubility of proteins, whereas acetylation tends to diminish it. Treating a protein with acetic anhydride causes amino groups to be acetylated:

$$\text{protein-NH}_2 + (\text{CH}_3\text{CO})_2\text{O} \rightarrow \text{protein-NH-CO-CH}_3 + \text{CH}_3\text{COOH}$$

This reaction converts the positive charge of the amino group to no charge. Maleic anhydride (Butler and Hartley, 1972) and citraconic anhydride (Atassi and Habeeb, 1972) are other related amino-specific reagents. They add negative charge to the protein, but the modification can be reversed by exposing the modified protein to acidic pH. Reversing a modification provides a way to ensure that functional changes attributed to the modification really were caused by it and not by incidental factors. It is wise, however, to determine early on whether the protein is stable to the reversal conditions, which can be harsh.

The following conditions are recommended as a starting point for succinylation. If sufficient protein is available, a range of conditions can be explored in parallel small-scale

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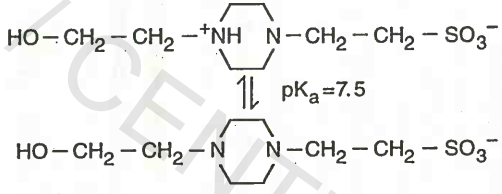
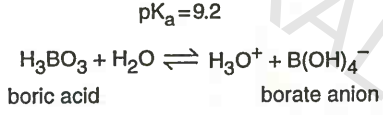
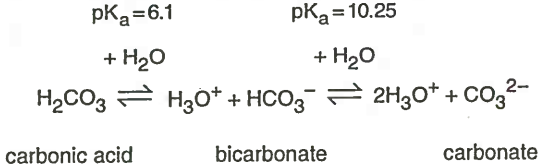
Table 15.2.3 Selected Buffer or Base Solutions Useful in Chemical Modification of Amino Groups

Buffer or base[a]	Relevant equilibria	Procedure
0.1 M HEPES, pH 8.0		Weigh 0.1 mol solid HEPES (zwitterion form) per liter H_2O and add it to ~90% of the final intended volume of H_2O. Adjust the pH to the desired value by adding suitable volumes of 5 M NaOH (*CAUTION:* Wear eye protection and gloves) while stirring the mixture and monitoring the pH with a pH meter. Add H_2O to final volume.
0.1 M sodium borate, pH 9.0		Weigh 0.1 mol boric acid per liter H_2O and add it to ~90% of the final intended volume. Adjust the pH to the desired value by adding 5 M NaOH (*CAUTION:* Wear eye protection and gloves) while stirring the mixture and monitoring the pH with a pH meter. All the boric acid will have dissolved when the pH reaches 9.0. Add H_2O to final volume.
0.1 M $NaHCO_3$ (not pH-adjusted), pH 8.3[b]		Dissolve 0.1 mol $NaHCO_3$ per liter of H_2O. The solution has a pH of 8.3. Use it as prepared; no further adjustment is needed.

[a]Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

[b]This solution is not a true buffer at pH 8.3, but provides an excess of a weak base (bicarbonate) to neutralize acid produced by the modification chemistry.

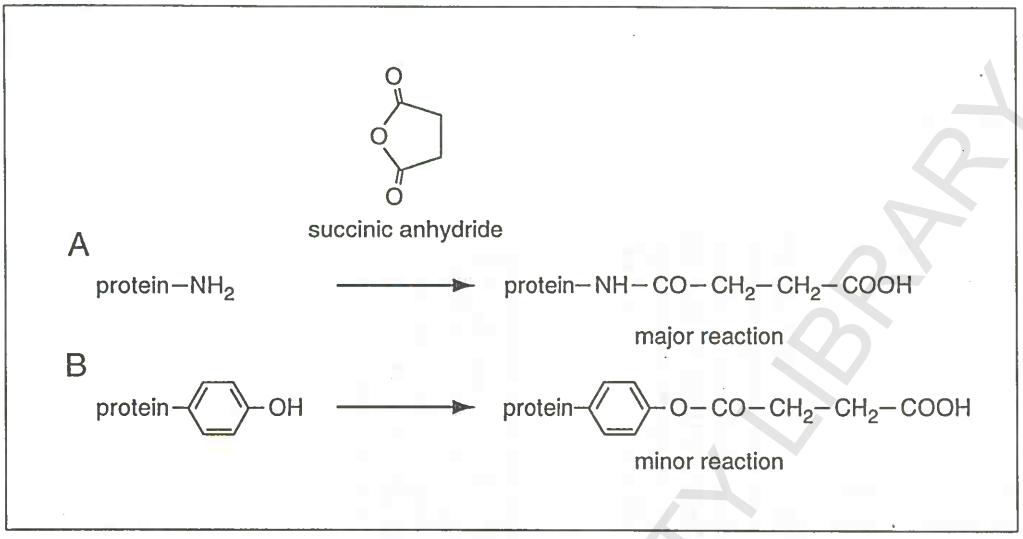


Figure 15.2.3 The principal reactions of succinic anhydride with proteins. (**A**) Succinylation of an amino group, which is normally the major result of treating a protein with succinic anhydride. (**B**) Succinylation of tyrosine, the minor reaction with a phenolic group of tyrosine; this reaction may be reversed by treating the protein with hydroxylamine·HCl at pH 7. Ionizable groups are drawn in their neutral state (amino as NH_2 and carboxyl as COOH).

experiments. Details of the procedure may have to be revised based on experience with a specific protein.

Materials

Succinic anhydride
1 to 10 mg protein/ml in 0.1 M sodium bicarbonate, pH 8.3
1 M NaOH
1 M hydroxylamine·HCl, pH 7.0

pH 6 to 10 dye-indicator pH sticks
Gel-filtration column containing chromatography medium (e.g., PD-10 column containing Sephadex G-25, Pharmacia Biotech)

Additional reagents and equipment for gel-filtration chromatography (*UNIT 8.3*) or dialysis (*UNIT 4.4* and *APPENDIX 3B*) and for mass spectrometry (see Chapter 16) or isoelectric focusing (*UNIT 10.2*)

1a. *To succinylate some, but not all, amino groups in the protein:* Calculate the amount of succinic anhydride that represents a 5-fold molar excess over ε-amino groups of Lys.

To obtain a 5-fold molar excess, the amount of succinic anhydride required is:

5 (mol protein) × number Lys/mol protein × formula weight of succinic anhydride = grams of reagent

For example, the mass of succinic anhydride giving a 5-fold excess over the Lys residues in 2 mg of a 20,000-Da protein that contains 10 Lys residues per mole of protein is determined as follows. (a) Determine the molar quantity of protein, 0.002 g/20,000 g mol^{-1} = 100 nmol protein; (b) multiply by 10 to determine the molar quantity of Lys residues in 100 nmol of this protein, 10 × 100 nmol = 1 μmol; (c) multiply by 5 to obtain the molar quantity of succinic anhydride required to give a 5-fold excess over Lys residues, 5 × 1 μmol = 5 μmol succinic anhydride; (d) calculate the mass of succinic anhydride (FW 100.1) corresponding to 5 μmol, (5 × 10^{-6} mol) × (100 g mol^{-1}) = 0.5 mg succinic anhydride.

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1b. *To succinylate all amino groups:* Calculate the amount of succinyl anhydride that represents a 20-fold molar excess over amino groups.

2. Add one-tenth the calculated amount of solid succinic anhydride to the protein with gentle shaking or stirring.

   This reaction is normally performed at room temperature, but it will proceed at 0°C.

   The succinic anhydride will dissolve within ~1 min while reacting with water or the protein.

3. Check the pH of the reaction mixture by placing 1 µl on the appropriate part of a pH 6 to 10 dye-indicator pH stick. If the pH has dropped to <8, add small amounts of 1 M NaOH until it returns to near the starting value.

   Succinic anhydride is a neutral compound, but its reactions with the protein and water liberate acidic groups and suppress basic amino groups. Consequently, the pH in the reaction mixture tends to fall. To maintain optimal conditions for modifying the amino groups, the pH must be kept at 8.0 to 9.0.

4. Add succinic anhydride in small portions at intervals of a few minutes until all of the required reagent is added. Continue to monitor and maintain the pH. After the final addition, incubate an additional 15 min.

5. Pass the reaction mixture over the gel-filtration column to remove residual low-mass reaction components.

   Dialysis (UNIT 4.4 and APPENDIX 3B) or diafiltration could be used as alternatives. The modified form of the amino groups is stable to these procedures.

6. Evaluate the success of the reaction.

   Mass spectrometry (see Chapter 16) is the best technique when full succinylation is desired; each succinylation adds 100.1 Da. Isoelectric focusing (e.g., on a Pharmacia PhastSystem apparatus or as described in UNIT 10.2) may be helpful with incomplete succinylations, as each charge changed from positive to negative shifts the isoelectric point. A colorimetric assay of amino groups using trinitrobenzenesulfonic acid (see Table 15.2.1) is also useful.

7. *Optional:* Reverse modifications at tyrosines by incubating the modified protein with 1 M hydroxylamine·HCl, pH 7.0, for 1 hr at 20°C.

REDUCTIVE METHYLATION

BASIC PROTOCOL 4

Reductive dimethylation of an amino group amounts to replacing its two hydrogen atoms with methyl groups, which converts a primary amine into a tertiary amine. The monomethyl amino group is formed first and readily undergoes a second round of modification to yield the final product. The resulting tertiary amine retains the ability to become protonated, making this modification a subtle probe of steric constraints about amino groups in the molecule. As the modified amino group is no longer an effective nucleophile, reductive methylation can be used to muzzle the reactivity of protein amino groups without otherwise greatly changing their properties.

The chemistry of each round of reductive methylation is itself a two-step process (see Fig. 15.2.4). First, reaction of formaldehyde with the protein results in reversible formation of a Schiff base (imine) adduct between the aldehyde and any protein amino group. Second, the modification is made permanent by the reducing (hydrogen-donating) action of sodium cyanoborohydride, which converts the imine to a carbon-nitrogen single bond. Unless the reagents are present in limiting supply, the resulting monomethylated protein amino group undergoes a further round of the same reaction to give the dimethylated form.

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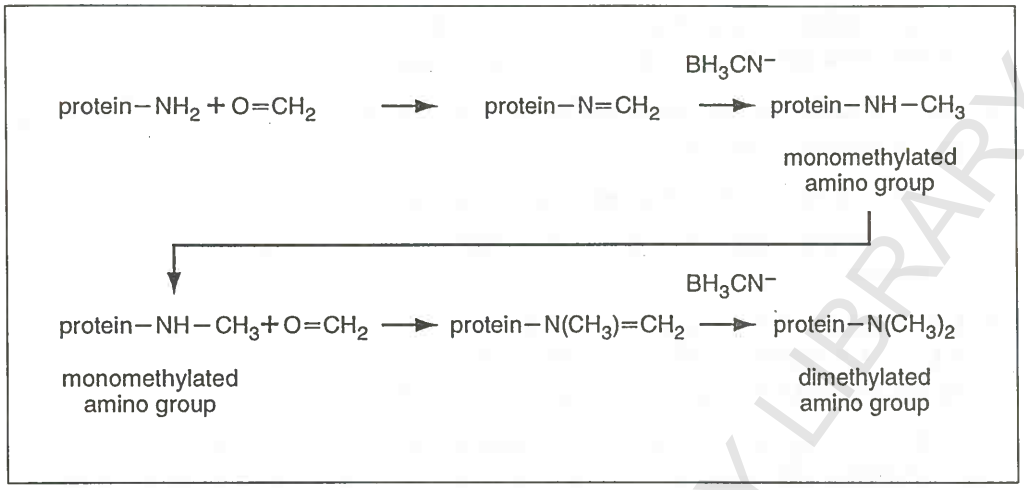

Figure 15.2.4 Reductive methylation of a protein amino group.

The overall extent of substitution varies with the conditions, as described in detail by Jentoft and Dearborn (1979). When consulting their excellent paper, note that the extent of modification in many of the examples approaches 1.0 methyl group per lysine, while the maximum possible extent of modification is 2.0 methyl groups per lysine. Higher concentrations of the reagents than those given by Jentoft and Dearborn (1979) are required when full dimethylation of all amino groups is sought, as may be the case when investigating structure-function relationships; for examples of such conditions, see Geoghegan et al. (1981). Comprehensive guidance for this procedure is available from the review by Jentoft and Dearborn (1983). ε-*N*-methyl-L-lysine and ε-*N,N*-dimethy-L-lysine are not normally present in acid hydrolyzates of proteins (although they do occur in rare cases), so standard chromatographic procedures for amino acid analysis require modification to permit determination of those amino acids (Elzinga and Alonzo, 1983).

Useful variations of this procedure have been developed. For example, aldehydes or ketones larger than formaldehyde can be used; the technique is then called, generally, reductive alkylation. This should not be confused with the approaches to sulfhydryl modification collectively known as reduction and alkylation (see *UNIT 15.1*). Other possibilities include the selection of modifying groups that can later be removed (Geoghegan et al., 1979; for a review, see Feeney, 1987). Reductive alkylation chemistry has also been used to immobilize protein or amino acid ligands for affinity chromatography, as in the Actigel ALD line of media from Sterogene Bioseparations.

Materials

1 to 10 mg protein/ml in 0.1 M sodium citrate, pH 6.0
37% formaldehyde (Aldrich)
Sodium cyanoborohydride ($NaBH_3CN$, Aldrich; see *CAUTION* below)
Methanol

Additional reagents and equipment for dialysis (*UNIT 4.4* and *APPENDIX 3B*) or gel-filtration chromatography (*UNIT 8.3*) and for mass spectrometry (Chapter 16) or amino acid analysis (*UNIT 3.2*)

CAUTION: Sodium cyanoborohydride is a highly toxic compound. Follow all precautions recommended in the Material Safety Data Sheet. Do not attempt to sniff the compound's odor, and be aware that exposing it to strong acid will result in the release of cyanide gas.

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1. Measure the volume of the protein/0.1 M sodium citrate solution. Calculate the volume of 37% formaldehyde required to give a final concentration of 20 mM and add to the solution.

   Formaldehyde has an M_r of 30, and a 37% solution therefore has a concentration of 12.3 M. A 1/100 dilution is 0.12 M; a further 1/6 dilution into the reaction mixture gives a concentration of 20 mM.

   It is important not to exceed the recommended level of formaldehyde in the reaction, as it is a cross-linking agent.

2. Calculate the volume of 1 M sodium cyanoborohydride solution that is required to give a final concentration of 10 mM in the reaction.

   Sodium cyanoborohydride has a formula weight of 62.84. A 1 M solution requires 63 mg/ml.

   As an alternative, a 1 M solution of reportedly highly purified $NaBH_3CN$ can be purchased from Sterogene Bioseparations (see SUPPLIERS APPENDIX) under the name of ALD Coupling Solution.

   If there is a particular desire to avoid the use of sodium cyanoborohydride, one of a number of amineboranes can be substituted (Geoghegan et al., 1981).

3. Weigh the hazardous sodium cyanoborohydride in the following manner. Tare a balance to zero with the tube to be used to prepare the solution. Working in a fume hood, transfer into the tube a quantity of sodium cyanoborohydride near the amount required. After closing the tube inside the hood, take the tube to the balance and weigh it again. Calculate the amount of methanol required to give a solution at the desired concentration, and carefully add it to the tube. Working in a fume hood, collect and rinse discarded spatulas and other equipment left over from the preparation of this solution.

   Use the solution immediately, as sodium cyanoborohydride begins to decompose as soon as it is dissolved.

4. Add methanolic sodium cyanoborohydride to the protein solution to a final concentration of 10 mM, i.e., a 1/100 dilution of the 1 M solution. Incubate 120 min at 22°C.

5. Separate the protein from low-mass reactants by dialysis (*UNIT 4.4* and *APPENDIX 3B*) or HPLC.

6. Use mass spectrometry (see Chapter 16) or amino acid analysis (*UNIT 3.2*) to determine the extent to which the amino groups of the protein have been methylated.

   Monomethylation of an amino group increases the mass of the protein by 14 Da, and dimethylation increases its mass by 28 Da.

RAPID DESALTING OF PROTEIN SAMPLES FOR ELECTROSPRAY MASS SPECTROMETRY

SUPPORT PROTOCOL

Mass spectrometry (Chapter 16) has revolutionized protein chemistry by making full molecular characterization readily attainable. Under favorable conditions, it can be used to measure the progress of a modification experiment with unmatched analytical accuracy. However, to reap the potential benefits of this technique, it is necessary to have a convenient means of preparing samples for analysis.

Mass spectrometry of biomolecules is an exercise with two major parts. The second is the mass analysis itself, but before that comes the requirement to generate a gas-phase ion of a large and labile analyte. Electrospray is one of a variety of methods for generating gas-phase ions. In this technique, a protein sample is sprayed as a population of droplets

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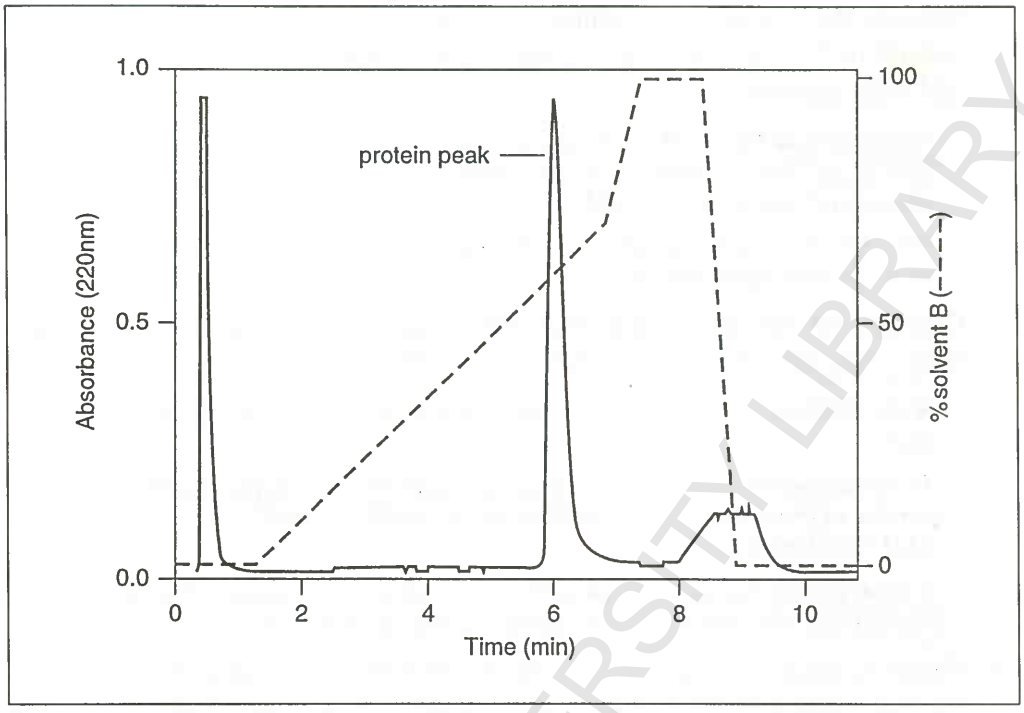

Figure 15.2.5 Rapid reversed-phase HPLC of a 61-kDa human recombinant intracellular enzyme (purified to homogeneity before this fractionation). The protein peak indicated by the arrow was collected, dried, and successfully analyzed by electrospray mass spectrometry.

into a compartment where it is exposed to high voltage. Evaporation of solvent from the sample droplets creates a destabilizing high charge density, and the droplets disintegrate in a process that makes multiply protonated gas-phase protein ions available for mass analysis. This can be achieved with 0.01% accuracy in a quadrupole-type spectrometer, i.e., to an accuracy of ± 4 Da in 40,000 Da.

The electrospray mode of sample introduction is very refractory to the presence of nonvolatile salt and buffer components in the sample. A convenient way to remove them is to use an HPLC system, consisting of a gradient pump system running at 1 ml/min, an injection device, and a UV-detector set to 220 nm. The system is configured to allow hand collection of peak fractions for rapid reversed-phase HPLC fractionation. The protein is retained on a 2.1-mm-i.d. × 3-cm-long reversed-phase HPLC column (e.g., Poros R2/H 2.1/30, PerSeptive Biosystems) for a short period while buffers and low-mass reagents are washed out in the injection peak. Elution is with an acetonitrile gradient using solvent A (0.1% [v/v] trifluoroacetic acid in water) and solvent B (0.085% [v/v] trifluoroacetic acid in 80:20 acetonitrile/water). This places the protein in a water/acetonitrile/trifluoroacetic acid solvent that is removed by evaporation. If the dried protein residue is visible (although this is not essential), there is usually enough protein for mass spectrometry (see Fig. 15.2.5 for the HPLC profile for a 61-kDa protein).

Although the HPLC column selected has an internal diameter of 2.1 mm (i.e., it is a narrow-bore column), the character of the packing allows it to run at flow rates >1 ml/min without excessive back-pressure. A flow rate of 1 ml/min is recommended. Gradient elution is performed over 6 min from 0 to 70% solvent B (i.e., 0% to 56% acetonitrile). First, a small portion of the protein is injected to determine that the protein can be recovered and to estimate the retention time; this will also give an impression of whether multiple protein peaks are to be expected. A quantity of the protein sample containing 1

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to 2 nmol is then injected, and the protein peak is collected by hand. Solvent is removed in a Speedvac evaporator.

NOTE: One nanomole of any protein is calculated on the basis of one microgram per kilodalton of relative mass, i.e., 1 nmol of a 20-kDa protein is 20 μg, and 1 nmol of a 100-kDa protein is 100 μg.

COMMENTARY

Background Information

This unit presents only a limited selection of approaches to amino-group modification. In general, the methods currently of greatest interest are those adaptable to bioconjugate formation, protein immobilization, and other technological applications. There is less attention to methods that appear useful only as probes of protein function, but they are well documented in the literature (Means and Feeney, 1971). Reactions in this category include carbamylation of amino groups with cyanate (also still significant as an incidental reaction of proteins exposed to high concentrations of urea, from which cyanate can form; Stark, 1967); formation of imidoamides (amidines) by reaction with imidoesters (Inman et al., 1983); and arylation of amino groups with a selection of reagents such as 1-fluoro-2,4-dinitrobenzene (Sanger reagent; Hirs, 1967), trinitrobenzenesulfonic acid (Fields, 1972), and dinitrobenzenesulfonic acid. Of this last group, trinitrobenzenesulfonic acid is the easiest to work with and readily modifies amino groups in aqueous solution at room temperature. Dinitrobenzenesulfonic acid reacts much more sluggishly under comparable conditions, but it can be useful for tagging small, heat-stable peptides with a dinitrophenyl group.

The classical modes of modification described in this unit now possess an importance rather different from that attached to them when they were new. Having once been among the most current tools for studying protein structure and function, they are now best viewed as prototypes for chemical modification schemes representing many ways to introduce new and useful groups into proteins. Succinimidyl esters, for example, are quite easy to prepare from carboxylic acids (see Brinkley, 1993), and they allow a growing range of nonnative structures to be made part of proteins by reaction with the protein amino groups.

Bifunctional reagents, a special category, are often directed toward amino groups at one or both of their two reactive sites (reviewed by Ji, 1983).

Critical Parameters

Proteins are subjected to chemical modification for a number of purposes, and the objective of the work defines the terms for all estimates of its success. In such a broad area, it is difficult to set practical guidelines for every case. The key discipline is to acknowledge that a chemical reaction is like a newly written computer program: it ruthlessly follows its own logic rather than the intentions of its creator. As a result, it is always important to consider whether the chemical modifications that have occurred (if any!) are actually those that were intended, and to ask whether any modifications that were not intended have also taken place.

If the reaction has been driven to achieve quantitative and exclusive modification of the amino groups in the protein, the product should be homogeneous and mass spectrometry (see Chapter 16) will be the appropriate and preferred way to characterize it. Partial modification gives a mixture of products, and the resulting molecules are more likely to have a mixture of properties. This is the classical flaw in the chemical modification approach. A mass spectrometric technique, such as MALDI-TOF MS (matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; *UNIT 16.2*), that accepts mixtures may still be useful for analyzing the results in these cases. Laboratories lacking mass spectrometers may find isoelectric focusing (*UNIT 10.2*) useful, and even SDS–polyacrylamide gel electrophoresis (*UNIT 10.1*) may be worth considering if the modification effects sufficient mass change in the protein to be detected. Finally, if a chromophore has been coupled to the protein molecule, absorption spectroscopy may furnish an estimate of the number of modifying groups introduced per molecule of protein.

Troubleshooting

Whatever the purpose of a modification experiment, failure to achieve the desired reaction is the most obvious form of trouble (see Table 15.2.4). Several likely explanations must always be considered. At the top of the list is

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Table 15.2.4 Troubleshooting Guide for Modification of Amino Groups

Problem	Possible cause(s)	Solution
Little or no modification is achieved	Reagent is already degraded when added to protein	Use fresh reagent. For reagents labile to hydrolysis, minimize or eliminate their exposure to aqueous solution before adding them to the protein.
	Reaction pH is inappropriate	Check the reaction pH after reagent has been added; it should be pH 8-9 in most cases. Monitor the pH and adjust it if necessary.
	The reagent is reacting with the buffer	If the buffer first chosen contains a primary or secondary amino group (e.g., Tris), change to a buffer that does not; e.g., HEPES, sodium bicarbonate, or boric acid/sodium borate.
Protein comes out of solution during modification reaction	Modification product is insoluble	Determine whether altered ionic strength or the addition of more or less organic solvent (e.g., acetonitrile) corrects the difficulty.
	Protein becomes insoluble when a large fraction of its amino groups are modified	If a lower level of modification can still be useful, reduce the amount of reagent and/or the reaction time.
	A solvent added with the reagent causes the precipitation	Test the effect of the solvent alone on protein solubility. If solvent causes the problem, minimize the amount added or use another solvent in its place.
Visible absorption spectrum of protein conjugate with a fluorescent reagent differs from that of free reagent	Multiple copies of strongly fluorescent groups (e.g., fluorescein) linked to a single protein molecule may achieve resonance energy transfer when irradiated	Distortion of the absorption spectrum is an artifact of placing multiple fluorescent groups in close proximity to each other. No unpredicted chemical change has occurred in the tagging group. Consult the literature.
Mass spectrometry of a heavily modified protein indicates that more equivalents of reagent were added than there are amino groups to accept them	Modification has occurred on sites other than amino groups, particularly tyrosyl residues	Incubate the modified protein with 1 M hydroxylamine·HCl for 1-5 hr at room temperature to reverse possible modifications at tyrosine, then recheck the product.

the possibility that the reagent is degraded and unsuitable for use. For example, succinimidyl esters are labile esters of carboxylic acids and can hydrolyze if exposed to water at neutral or basic pH before use. The risk of hydrolysis is not present when reagents are dissolved in dry aprotic solvents such as acetonitrile or dimethylformamide. Long-term exposure to trace levels of water may be effective in degrading reagents, so older or poorly stored supplies are always suspect.

Another likely error is inclusion in the reaction medium of amino-containing compounds that compete with protein amino groups for the modifying group. Apart from the hazards, already mentioned, of using amine-containing buffer components such as Tris or glycine, any compound with a primary or secondary amino group is a potential competitor with the protein amino groups.

The reaction conditions must also be reconsidered when trouble occurs. Was the pH at or near the desired value? See Scopes (1994) for a superb discussion and practical advice on choosing and making buffers. Did the pH remain at this value after the time allowed for reaction has been completed? If pH is satisfactory, then temperature must also be considered.

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The reactions described in this unit are fairly fast at room temperature (~20°C), but some other procedures for amino-group modification may require gentle heating. In particular, dinitrophenylation of amino groups using dinitrobenzenesulfonic acid is a slow reaction that requires temperatures >40°C. This may make it unsuitable for use with many proteins, but it can be used successfully with peptides.

Anticipated Results

The amino groups of proteins are easy to modify, and failure of these procedures is usually caused by overlooking some technical detail. A harder task is to predict accurately all the properties of a modified protein. Even a single hydrophobic fluorescent tag, for example, can seriously diminish the solubility of a protein that was easily handled in its native form. This type of uncertainty is part of any chemical experiment; it simply calls for vigilance and a guarded attitude by way of response, accepting that there may be unforeseen consequences when the charge distribution on a protein's surface is varied or novel structures are introduced. As the native folded form of a protein typically enjoys only a marginal advantage in stability relative to many unfolded structures, it is not surprising that changing the structure sometimes has consequences that go beyond those first intended.

Time Considerations

Most modification reactions should be completed in ≤2 hr, and fast methods such as gel-filtration chromatography (*UNIT 8.3*) can be used to avoid long delays in the follow-up. Microconcentrators such as the Centricon and related lines (Amicon) are also valuable tools for reducing sample volumes or achieving buffer exchanges in relatively short order, but note that their internal surfaces may need to be treated with polyethylene glycol to reduce adsorptive losses of protein (see manufacturer's instructions and *UNIT 4.4*). The results of most chemical modification experiments should be well understood within 24 hr of their inception.

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Comprehensive coverage of modifying agents.

Contributed by Kieran F. Geoghegan
Pfizer, Inc.
Groton, Connecticut

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MASS SPECTROMETRY

16

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Overview of Peptide and Protein Analysis by Mass Spectrometry

WHY IS MS AN ESSENTIAL TOOL IN PROTEIN STRUCTURE ANALYSIS?

Mass spectrometry (MS) is now regarded as an indispensable tool for peptide and protein primary structure analysis. The increasingly widespread use of this technique is due in large part to its ability to solve structural problems not easily handled by conventional protein chemistry techniques. MS can provide accurate molecular weights for peptides and proteins with masses up to 500,000 Da using only a few picomoles (tens of femtomoles in favorable cases) of material. The accuracy achieved by MS is frequently better than 0.01% of the calculated mass (see Table 16.1.1). This means, for example, that the experimentally determined M_r of a 25-kDa protein will be ±2.5 Da of the actual mass. In contrast, molecular weight estimates obtained by SDS-PAGE have accuracies of 5% to 10%; moreover, the mobility of the protein in the gel can be grossly affected (up to 50%) by the presence of covalent modifications such as lipids and carbohydrates. The accuracy of protein molecular weight estimates obtained by other methods, such as sedimentation velocity and gel permeation, can also be affected by the presence of lipid or carbohydrate. For correction and interpretation of this data, a molecular weight derived by mass spectrometry is needed (Hensley et al., 1994).

Unlike these other techniques, mass spectrometry provides molecular mass information independent of any modifications to the structure (such as glycosylation and phosphorylation). A mass difference between the observed and the expected mass of the protein or a peptide suggests the presence of post-translational modification or proteolytic processing, and the mass difference will be the starting point for defining the structure of the modification. A difference of +42 Da, for example, together with the lack of a free NH_2-terminus, is indicative of the presence of an α-*N*-acetyl group. Similarly, a difference of +80 Da suggests the presence of a phosphate or sulfate moiety, and a cluster of molecular ion peaks separated by 162, 203, 291, or 146 Da indicates glycoforms differing by the presence of hexose, *N*-acetylhexosamine, *N*-acetylneuraminic acid, or deoxyhexose (see Tables A.1A.2 and A.1A.3 in *APPENDIX 1A*). Thus molecular weight information provides a useful measure of the integrity, purity, and overall state of modification of a peptide or protein. When evaluated in the context of other available structural data, the molecular mass information alone can be used to solve a variety of peptide and protein structural problems.

The ability of MS to determine the molecular weights of peptides in mixtures (such as result from a proteolytic digest of a protein) with better than 0.01% accuracy forms the basis of the mass spectrometric peptide mapping strategy, a rapid and highly efficient method to verify the fidelity of translation of protein sequences deduced from DNA or cDNA sequencing. This can be done either by direct analysis of unfractionated mixtures (Billeci and Stults, 1993) or on-line with liquid chromatography (Hess et al., 1993; Carr et al., 1993; Kassel et al., 1994) or capillary electrophoresis (Wahl et al., 1992; Smith et al., 1996; Foret et al., 1994).

Mass spectrometry also has the ability to provide amino acid sequence information on peptides using methodology generically referred to as tandem mass spectrometry (MS/MS). Using MS/MS, complete or partial sequence information may be obtained at the femtomole to picomole level for peptides containing up to 25 amino acid residues. In favorable cases some internal sequence data can often be obtained for peptides up to 30 or 40 residues. Unlike Edman sequencing, MS can provide this type of sequence information even if the peptides are present in complex mixtures, and, more importantly, even if they are modified. Methodologies such as Edman degradation and amino acid analysis have serious limitations with regard to characterizing structurally modified proteins and peptides, because many commonly occurring modifications are lost or destroyed by the harsh cleavage and derivatization conditions employed (Uy and Wold, 1977; Wold, 1981; Wold and Moldave, 1984; Carr et al., 1991). Furthermore, the sensitivity and speed of MS-based sequencing now exceeds that of Edman-based sequence analysis (Hunt et al., 1992; Wilm et al., 1996).

Vast amounts of cDNA sequence data, obtained by grand-scale sequencing of the human and other genomes, are presently flooding into databases. However, little is known about the

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Table 16.1.1 Key Ionization Methods for Biomolecule Analysis

Ionization method	Tolerance for buffers, salts, and detergents	MW range	Mass assignment accuracy	Compatible with on-line LC/CE-MS
Electrospray ionization (ESI)	Low tolerance (≤20 mM) for nonvolatile buffers, alkali metal salts, detergents; on-line LC-MS used for contaminant removal	≤150 kDA	±0.005%–0.01% to 50 kDA ±0.02%–0.03% to 150 kDA	Yes
Matrix-assisted laser desorption/ionization (MALDI)	High tolerance (≥50 mM) for alkali metal salts, phosphate, urea, etc.; tolerant of some nonionic detergents; intolerant of SDS	≥350 kDA	±0.01%–0.05% to 25 kDA ±0.05%–0.3% to 300 kDA	No

functions of most of the proteins the various genes encode, nor is much known about the processed states of the expressed, mature forms of the proteins. In contrast, the protein content of a cell, referred to as the proteome (Kahn, 1995; Wilkins et al., 1995), can be studied by protein differential display using two-dimensional gel electrophoresis (*UNIT 10.4*), immunoprecipitation, and related procedures, allowing physiologically relevant proteins to be recognized by changes in their expression levels in response to stimuli. Because of its speed and sensitivity, mass spectrometry, through the use of strategies that employ molecular weight and/or partial sequence information to search protein databases, has emerged as the primary technology for identifying these proteins (Henzel et al., 1993; Mann et al., 1993; Pappin et al., 1993; Wilm and Mann, 1994; Eng et al., 1994).

This introductory overview has been designed with several goals in mind. The first and perhaps the most important one is to try and dispel the still widely held, but now mistaken, belief that mass spectrometry is beyond the technical resources of the typical laboratory or facility engaged in the structural characterization or synthesis of peptides and proteins. Over the last five years, mass spectrometers and associated data systems have become available that can be operated by anyone capable of running a modern amino acid analyzer or Edman sequencer, yet are powerful enough to handle the most demanding analyses that the peptide or protein investigator might require. As a result, it is no longer necessary to be a "card-carrying" mass spectroscopist to use the instruments and techniques of MS effectively and to apply it successfully to one's own research program. The remaining goals of this article are to familiarize peptide and protein chemists with the types of mass spectrometers that are appropriate for the majority of their analytical needs, to describe the kinds of experiments that can be performed with these instruments on a routine basis, and to discuss the kinds of information that these experiments provide. The emphasis here is on established tools and techniques that can realistically be used for problem solving. As a result, many useful instruments and techniques employed by the trained mass spectroscopist are not discussed here, because they do not satisfy these criteria or are not yet widely available. A few of these are briefly mentioned at the end of this introductory overview. The units that follow this overview will further discuss many of the principles introduced here and provide detailed protocols and applications of the methods. The overview concludes with a tutorial discussion on the meaning and practical importance of a number of fundamental experimental and performance-related issues that have important implications for interpretation and use of mass spectral data, such as mass accuracy and resolution.

More comprehensive discussion of mass spectrometry of biological molecules may be found in several recent books (Burlingame and McCloskey, 1990; McCloskey, 1990; Burlingame and Carr, 1996) and review articles (Carr et al., 1991; Biemann, 1992; Chait and Kent, 1992; Aebersold, 1993; Wang and Chait, 1994; Mann and Wilm, 1995; Williams and Carr, 1995; Mann and Talbo, 1996). The biannual review of mass spectrometry in *Analytical Chemistry* by Burlingame and colleagues provides a particularly good annotated bibliography of the recent literature on techniques and application of MS in the biological sciences

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(Burlingame et al., 1994). Mass spectrometry information is also rapidly proliferating on the World Wide Web; Table 16.2.4 contains a useful listing of sites that provide information about MS.

WHAT IS MS?

Mass spectrometry is a powerful analytical technique for forming gas-phase ions from intact, neutral molecules and subsequently determining their molecular masses. All mass spectrometers have three essential components: an ion source, a mass analyzer, and a detector. Ions are produced from the sample in the ion source using a specific ionization method (see discussion of Key Ionization Methods and Related Ancillary Techniques). The ions are separated in the mass analyzer based on their mass-to-charge (m/z) ratios and then detected, usually by an electron multiplier. The data system produces a mass spectrum, which is a plot of ion abundance versus m/z. Except in the case of electrospray mass spectrometry (where the ion source is at atmospheric pressure), the ion source, mass analyzer, and detector are usually situated inside a high-vacuum chamber (pressure between 10^{-8} and 10^{-4} Torr).

KEY IONIZATION METHODS AND RELATED ANCILLARY TECHNIQUES

The decade of the 1980s marked a revolutionary period in the development of "soft ionization" techniques for the analysis by mass spectrometry of large, polar, nonvolatile molecules (Carr et al., 1991). These ionization techniques produced primarily intact protonated molecular ions of peptides and proteins. In the early 1980s, fast atom bombardment (FAB) and, to a lesser extent, plasma desorption (PD) mass spectrometry were used extensively for structural characterization of peptides and small proteins. In 1988, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry were first shown to be useful for the analysis of peptides, proteins, carbohydrates, and oligonucleotides. Within a few years of their introduction, ESI and MALDI techniques became the MS methods of choice for biopolymers analysis, largely supplanting FAB-MS and PD-MS. For this reason these techniques will be the focus of this overview. The capabilities and sample requirements of these two MS techniques are compared in Table 16.1.1. The performance specifications in the table are those that can be routinely achieved by trained operators and do not represent the ultimate performance of each technique.

Several factors account for the widespread acceptance of ESI-MS and MALDI-MS. Both ionization methods (see detailed descriptions below) are optimally combined with relatively low-cost, simple-to-operate mass analyzers. A quadrupole mass filter is typically used for ESI-MS, whereas a time-of-flight (TOF) analyzer is commonly employed for MALDI-MS. The sensitivity of both methods is in the low- to subpicomole range, and both are capable of analyzing molecules with a wide range of molecular weights (Table 16.1.1). The methods have many analytical capabilities in common, but do differ in a number of respects that are of practical significance. Both techniques work best with clean (salt- and detergent-free) samples, but MALDI is capable of providing information directly on relatively dirty samples. Both methods can provide molecular weight information for large proteins, but the information is invariably easier to obtain and more sensitive with MALDI-MS. In the molecular weight range from 5 to 50 kDa, however, analysis by ESI-MS usually provides much more accurate molecular weight measurements. ESI-MS in this molecular weight range also typically exhibits better mass resolution than MALDI, which permits detection of protein variants that differ only slightly in mass. These practical differences between the techniques, and their impact on the information that can be obtained about a protein structure, are discussed below.

In addition to the development of the ionization approaches described above, a number of key enabling technologies that have significantly improved the performance and sensitivity of ESI-MS and MALDI-MS have been introduced in the last few years. These technologies have moved MS onto the center stage of protein discovery and characterization. The first such advance has been the development and refinement of microscale desalting procedures such as efficient liquid-liquid extraction for peptides produced by in-gel digestion (Shevchenko et al., 1996), micropacked glass capillaries for cleanup of 1 to 5 µl of sample (Houthaeve, et al., 1995), and microscale high-performance liquid chromatography (HPLC) employing columns with internal diameters ≤1mm (Zhang et al., 1995). Second has been the development of ultra-low-flow (<50 nl/min) electrospray sources that enable 1 to 2 µl of a dilute sample containing femtomole per microliter concentrations of peptides to be an-

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alyzed for periods of >60 min (Wilm and Mann, 1996). The long duration of analysis permits many experiments, including conventional MS experiments and MS/MS, to be carried out on a single sample loading. A third development has been the introduction of novel scanning methods whereby MS can be used to selectively detect specific protein modifications (Carr et al., 1993; Huddleston et al., 1993a,b). Fourth is the development of the so-called post-source decay (PSD) experiment (Kaufmann et al., 1994), which provides the ability to mass select particular peptide molecular ions and to analyze the metastable fragment ions from this precursor that are formed during the MALDI process. Like the ESI tandem MS experiment, PSD can provide a substantial amount of peptide sequence information (see section entitled What Is Tandem MS?). Finally, a variety of computer-based searching strategies have been developed whereby known proteins can be identified from protein and DNA databases by searching MS-derived molecular weights with and without partial sequence information (Henzel et al., 1993; Mann et al., 1993; Pappin et al., 1993; Wilm and Mann, 1994; Eng et al., 1994; Hall et al., 1996).

WHAT IS TANDEM MS?

In tandem mass spectrometry (commonly referred to as MS/MS), two consecutive stages of mass analysis are used to detect secondary fragment ions that are formed from a particular precursor ion. The first stage serves to isolate a peptide precursor ion of interest based on its *m/z*, and the second stage to mass analyze the product ions formed by spontaneous or induced fragmentation of the selected precursor ion. Interpretation of the product-ion spectrum provides sequence information for the peptide selected. The manner in which product ions are produced and detected is different in MALDI and ESI mass spectrometry, and therefore is described in the following sections that are devoted to these specific techniques.

MS/MS provides information that is highly complementary to that provided by Edman degradation; in addition, MS/MS has several advantages relative to Edman degradation for determining peptide sequence. First, MS/MS can produce sequence information for blocked or otherwise modified peptides that are either difficult or impossible to sequence by Edman degradation (for example, see the MS/MS spectrum of the phosphopeptide in Fig. 16.1.6). Second, the ability to individually select and fragment peptides in mixtures eliminates the need for extensive purification of peptides prior to sequence analysis. Third, MS-based peptide sequencing is faster than Edman sequencing because it is not a stepwise process. All of the structural information, in the form of fragment ions, is presented in a single mass spectrum acquired in a single experiment. Finally, MS-based approaches are now at least as sensitive as Edman degradation (e.g., 1 to 5 pmol of peptide or protein is required), and state-of-the-art MS-based sequencing by either ESI-MS or MALDI-MS is capable of providing sequence information at the 50- to 250-fmol level (Hunt et al., 1992; Wilm et al., 1996).

Peptide fragment ions are produced in the mass spectrometer primarily by cleavage of the amide bonds (—CO-NH—) that join pairs of amino acid residues. The most commonly observed fragment ions and their nomenclature are shown in Figure 16.1.1, and a list of commonly observed low-mass fragment ions indicative of the presence of particular amino acid residues is presented in Table 16.1.2. The fragmentation of peptides in MS has been well described (Hunt et al., 1986; Biemann, 1988, 1990; Falick et al., 1993). The interested reader is referred to these studies and references therein for more detailed discussions of fragmentation and its possible mechanisms. The difference in mass between adjacent sequence ions of the same type defines an amino acid. Of the twenty commonly occurring amino acids, sixteen have unique masses, Leu and Ile have exactly the same mass, and Lys and Gln have nearly the same mass (Table A.1A.1 in *APPENDIX 1A*). A mass difference between two adjacent sequence ions of the same fragment type that does not correspond to the mass of an amino acid residue may represent the presence of a post-translational modification. A list of common post-translational modifications and their masses is given in Table A.1A.3 in *APPENDIX 1A*.

Two caveats to the above discussion on MS/MS should be noted. First, although it is generally very easy to obtain the internal sequence of a peptide by this procedure, it is often difficult to determine the order of the first two and sometimes the last few residues in the peptide without additional experiments. Several methods involving reanalysis by MS/MS after chemical derivatization have been developed to help sort out the sequence at the ends of the peptide when required (Sherman et al., 1995; Gulden et al., 1996). Alternatively, there are several sequence ladder approaches, using either chemical derivatives or peptidases, that can be useful for identifying the N- and C-ter-

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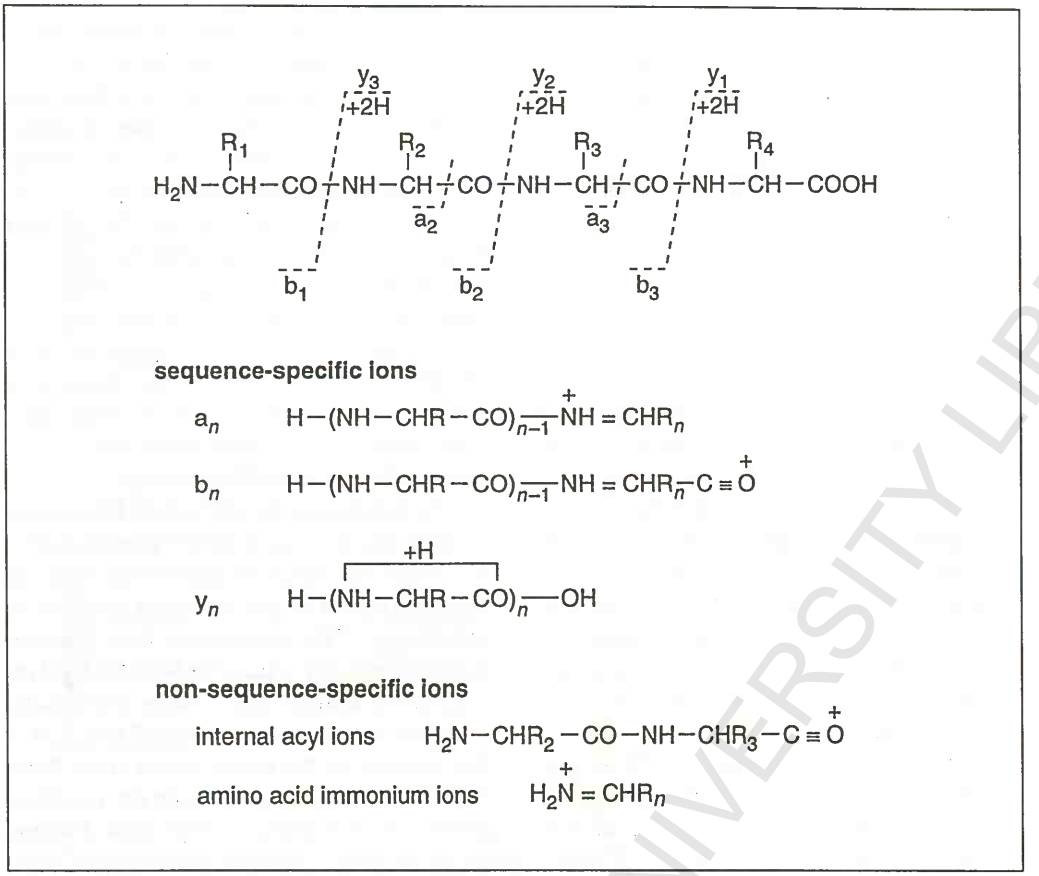

Figure 16.1.1 Nomenclature for peptide fragment ions. R represents the variable amino acid side chain (see *APPENDIX 1A*). The non-sequence-specific ions are designated by the single-letter amino acid codes (Table A.1A.1) for the R groups they include.

Table 16.1.2 Common Low-Mass Ions Characteristic of Amino Acids[a]

m/z	Amino acid present
70, 87, 100, 112, 129	Arginine
70 (w/o 87, 100, and 112)	Proline
72	Valine
84 (w/o 101)	Lysine
86	Leucine or isoleucine
101 (w/o 84)	Glutamine
102	Glutamic acid
104	Methionine
106	Pyridylethyl cysteine
110	Histidine
120	Phenylalanine
133	Carbamidomethylated cysteine
134	Carboxymethylated cysteine
136	Tyrosine
147	Acrylamide-modified cysteine
159	Tryptophan
216	Phosphotyrosine

[a]Underlining indicates more abundant ions; w/o, without.

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minal residues (Chait et al., 1993; Bartlet-Jones et al., 1994; Thiede et al., 1995; Patterson et al., 1995). The second caveat is that, unlike Edman degradation, MS cannot at present be used reliably to determine the NH_2-terminal sequence of proteins directly, although several successful attempts have been made (Speir et al., 1995).

MALDI-MS

Matrix-assisted laser desorption/ionization (MALDI)-MS as commonly practiced today was first described by Karas and Hillenkamp (1988). They demonstrated that by cocrystallizing analyte molecules with a large molar excess of a small, UV-absorbing organic acid, large proteins such as serum albumin could be desorbed and ionized by short, intense pulses from a UV laser and detected using a TOF mass spectrometer. Refinement of the technique was rapid. New laser wavelengths and correspondingly optimized matrices were introduced (Beavis et al., 1992). In less than two years, proteins >100 kDa were being routinely analyzed with subpicomole sensitivity (Hillenkamp et al., 1991) and molecular weights could be assigned with an accuracy of ~0.1%. Beavis and Chait (1990) demonstrated that for small proteins (<30 kDa) mass accuracies on the order of 0.01% to 0.02% could be achieved by coadding peptides or proteins of known mass to the sample to serve as internal mass reference standards (Beavis and Chait, 1990). This is 2 to 3 orders of magnitude more accurate than SDS-PAGE.

MALDI-MS has also been shown to be extremely useful for the analysis of peptides (Billeci and Stults, 1993). Unlike ESI, MALDI produces predominately singly charged molecular ions from peptides and proteins (although larger proteins can produce multiply charged ions as well; see Fig. 16.1.8), making the analysis of mixtures very much more straightforward. The ease of spectral interpretation combined with femtomole sensitivity and a tolerance for many of the common biological buffers makes MALDI an obvious choice for the analysis of unfractionated enzyme digests. Recent developments in the use of MALDI-MS for the analysis of peptides and proteins have been reviewed (Mann and Talbo, 1996; Wang and Chait, 1994).

The MALDI process can result in extensive fragmentation of the sample. Decomposition that occurs in the ion source, upstream from the field-free region of the flight tube, is referred to as "prompt fragmentation" and can be observed in a linear spectrum. Ions formed by prompt fragmentation are usually of low abundance, and are formed when very high laser irradiance is used. For example, disulfide bonds have been demonstrated to undergo prompt fragmentation to yield the free peptides (Zhou et al., 1993; Patterson and Katta, 1994). Fragmentation that occurs after the source, in the flight tube, is referred to as metastable decay or post-source decay (Kaufmann et al., 1994). These fragment ions, which are often abundant, cannot be observed in a linear TOF instrument but can be observed with the use of a reflectron. As described below, they can be very useful for peptide sequencing.

The mechanism by which MALDI operates is still a matter of some debate (Dreisewerd et al., 1995). The one point upon which everyone agrees, however, is that the matrix is critical to the success of the experiment. In its simplest form, sample preparation for MALDI involves mixing the sample with a large (10^4) molar excess of matrix and applying 0.5 to 2.0 µl of this solution to the target, where it air dries. Once in the mass spectrometer, the sample is bombarded with photons of UV light. The matrices, all compounds that are strongly UV-absorbing at the designated wavelength, serve three major functions in the experiment. First, the large excess of matrix separates the analyte molecules from one another, thereby reducing intramolecular interactions. Second, the matrix rapidly absorbs large amounts of energy from the incoming photons, resulting in an explosive breakdown of the matrix-analyte lattice. This explosion sends both matrix and analyte molecules into the gas phase. Third, the matrix is necessary for ionization of the analyte molecules. Transfer of protons from the matrix to the peptide and protein molecules is believed to occur via gas-phase reactions in the dense cloud that forms above the target during laser irradiance. The choice of matrix depends on the irradiance wavelength and the type of sample to be analyzed. A N_2 laser operating in the UV at 337 nm is the most typical laser in use on commercial MALDI instruments. Matrices that have been shown to be routinely useful at 337 nm are 3,5-dimethoxy-4-hydroxy-*trans*-cinnamic acid (sinapinic acid), 2,5-dihydroxybenzoic acid, and α-cyano-4-hydroxy-*trans*-cinnamic acid (Hillenkamp et al., 1991; Beavis et al., 1992). Lasers that operate in the infrared at 2.94 µm have also been shown to be useful in MALDI applications, particularly for the analysis of proteins directly from gels or blots (Strupat et al., 1994). MALDI has been dem-

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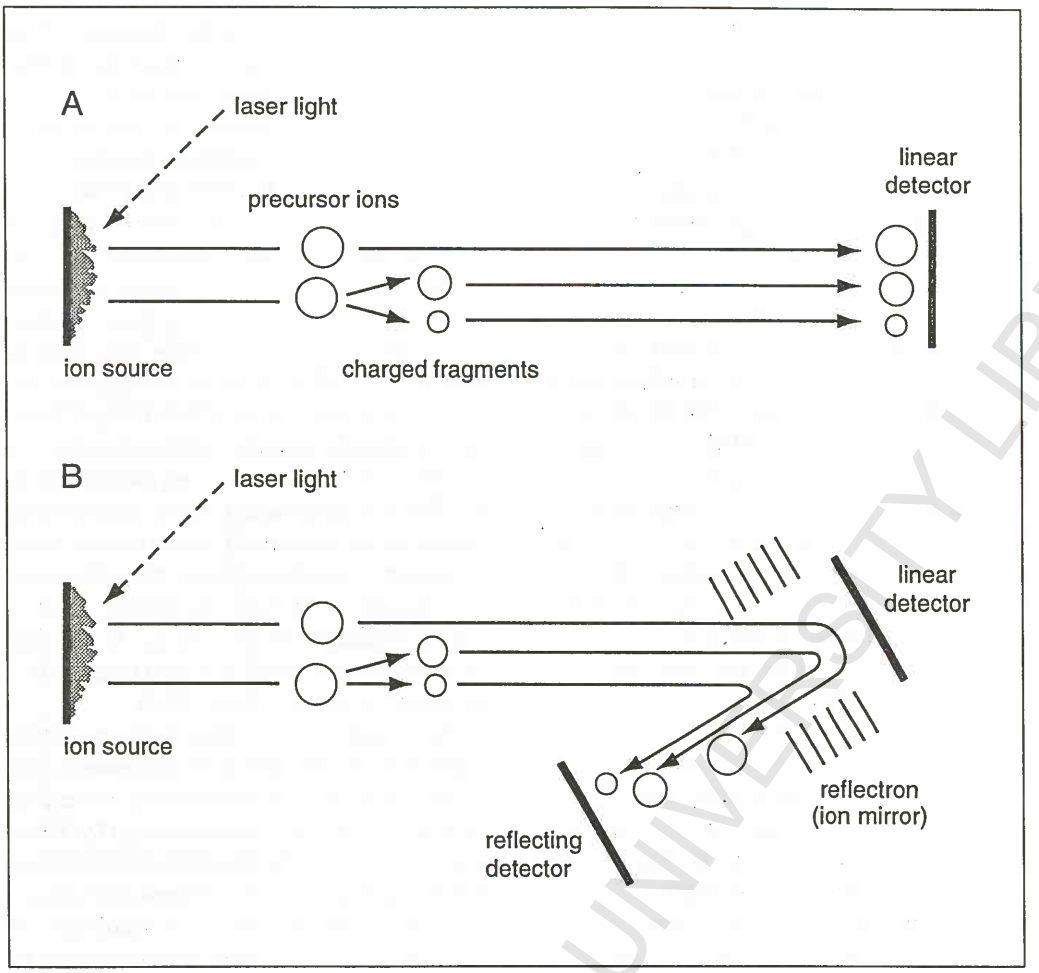

Figure 16.1.2 Schematic diagrams of linear (**A**) and reflectron (**B**) MALDI-TOF mass spectrometers. The flight paths of precursor and fragment ions in each instrument are shown.

onstrated on magnetic-sector (Annan et al., 1992; Medzihradszky et al., 1996), ion-trap (Qin and Chait, 1995), and Fourier-transform ion-cyclotron resonance (Wu et al., 1995) mass spectrometers, but by far the most common application of MALDI has been on TOF instruments.

Typical TOF mass spectrometers can be classified into two broad categories, linear and reflector-type instruments. A simple linear instrument (Fig. 16.1.2, panel A) consists of an ion source, where the MALDI process takes place; a flight tube, where ions of different masses are separated from one another; and a detector. All of these components, with the exception of the laser, are housed in a high vacuum maintained at 10^{-5} to 10^{-8} Torr. After ions are formed by the MALDI process, they are accelerated out of the source under the influence of a strong electric field. All of the ions have essentially the same final kinetic energy upon entering the flight tube; thus, the arrival time of a given ion at the linear detector will vary depending on its mass. These relationships are mathematically expressed by classical equation for kinetic energy (here expressed in two forms), where E is the kinetic energy of an ion, m is its mass, and v is its velocity:

$$E = \tfrac{1}{2}\, mv^2$$

$$v = (2E/m)^{0.5}$$

These equations tell us that because all ions of the same charge have been accelerated to the same energy, their velocities (and therefore, their flight times) will be inversely proportional to the square root of their molecular masses. Thus ions of greater mass will travel slower than lighter ones and will therefore reach the detector later.

Simple linear MALDI-TOF instruments are rather low-resolution mass spectrometers, operating at a resolving power of ~450 to 600 for peptides and 50 to 400 for proteins. For peptides, this means that one cannot measure the distribution of the various naturally occurring

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isotopes for each peptide, or separate peptides which differ in mass by only a few daltons. It also means that given a resolution (R) of 400, it will be difficult to resolve two proteins >30 kDa that differ only by an initiator Met residue (e.g., see Fig. 16.1.8). These issues are considered in more detail in the discussion of Fundamentals of Mass Measurement Accuracy and Mass Resolution.

Several important considerations relating to the production of ions in the MALDI source limit the resolving power of linear TOF instruments. The most important of these is the initial kinetic energy spread of individual ion populations: as a result of this spread, ions of any particular mass will, after reaching final accelerating voltage, exhibit a spread in velocities. This means that various members of the same ion population will arrive at the detector at slightly different times, and that in turn gives rise to a broader peak for each ion than would be observed if there were no initial kinetic energy spread. Because the initial energy spread is mass dependent, peaks for higher-mass ions will be disproportionally broader than those for lower-mass ions. Resolution in a linear TOF instrument can be improved by increasing the length of the flight tube. This enhances the time dispersion of ions of different *m/z*; unfortunately, it also increases the spread of arrival times for ions of the same *m/z* due to the initial kinetic energy distribution. This energy spread is typically reduced in magnitude by increasing the final accelerating voltage.

A more effective way to correct for energy distributions is through the use of an ion mirror, or reflectron. A reflectron TOF instrument (Fig. 16.1.2, panel B) corrects for the initial energy spread by acting as an energy-focusing device. The reflector works by slowing an ion down until it stops (the back of the reflector is at a voltage slightly higher than the source-accelerating voltage), turning it around, and then reaccelerating it back out to a second detector. Ions with an initial kinetic energy (and velocity) slightly lower than full accelerating potential will not penetrate the reflector as deeply, and will therefore turn around sooner, catching up to those ions with full kinetic energy. Ions with slightly greater energies (and thus higher velocities) will penetrate more deeply into the reflector, be turned around later, and have their flight times retarded, allowing the other ions to catch up. All of this causes ions of a given mass-to-charge ratio to be spatially focused into packets having flight times that are closer together, thus improving the resolution. This improvement in resolution is illustrated in Figure 16.1.3, which shows the molecular ion region of the linear (panel A) and reflector (panel B) spectra of a synthetic tyrosine phosphorylated peptide TRDIYETDpYYRK. The isotopically resolved ion cluster in the reflector spectrum shows the contributions of all of the isotopes of C, H, N, O, and P that make up the elemental composition of this peptide. The improvement in resolution is most noticeable at masses of ~3000 and lower. For larger molecules, where the resolution is no longer sufficient to provide separation of the isotopes, the natural width of the unresolved isotopic envelope has a large influence on the apparent resolution of the instrument, as explained in the discussion of Fundamentals of Mass Measurement Accuracy and Mass Resolution. In addition to resolution, mass accuracy is also improved when spectra are recorded in the reflector mode (Vorm and Mann, 1994).

Tremendous improvement in the resolution achievable on MALDI-TOF instruments has recently been demonstrated using a focusing technique developed many years ago by Wiley and McLaren (1953). This method, referred to as time-lag focusing or "delayed extraction," has been shown to provide a resolution of ~2000 to 4000 (full-width, half-maximum; or FWHM) in linear mode for peptides and resolution of ~3000 to 6000 FWHM in reflecting mode, on a 2-meter-long instrument (Brown and Lennon, 1995; Whittal and Li, 1995; King et al., 1995; Vestal et al., 1995). In a delayed extraction source, ions are created in a field-free region and allowed to spread out before an extraction voltage is applied and the ions are accelerated into the drift tube. The delayed extraction also probably limits the number of collisions the ions undergo on their way out of the source, thereby reducing additional peak broadening caused by metastable decomposition. Mass accuracy with the use of an internal reference standard may also be improved with the use of the delayed extraction source.

Assigning an accurate *m/z* value to an ion whose flight time has been measured requires that the mass spectrometer be properly calibrated. This is usually accomplished by measuring the flight times of two compounds whose molecular weights are accurately known. In practice, it is usually best to select two compounds with molecular weights that bracket the mass range over which samples are to be examined. For molecules >10,000 Da, the $[M+H]^+$ and the $[M+2H]^{2+}$ ions of a well-

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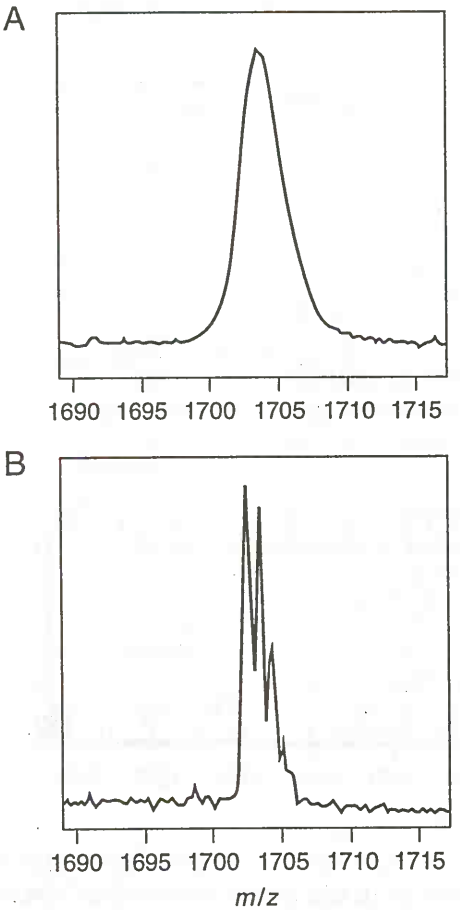

Figure 16.1.3 Molecular ion region from MALDI spectra of the peptide TRDIYETDpYYRK (monoisotopic M_r = 1701.8) recorded in the (+) ion mode. Spectra were recorded in (**A**) the linear mode, where the average mass is measured at the centroid of the unresolved isotopic cluster, and (**B**) the reflector mode, where the monoisotopic mass is measured at the centroid of the first peak in the cluster. This peak contains the lightest isotopes of the elements present (see discussion of Fundamentals of Mass Measurement Accuracy and Mass Resolution).

characterized protein standard work very well for calibration (see *UNIT 16.2*). Application of an internal calibration, where the calibrant peaks are present in the same spectrum as the sample, can provide accuracies of 0.01% to 0.05% (e.g., 0.1 to 0.5 Da at m/z = 1000). Internal calibration is sometimes experimentally difficult owing to the need to adjust the amount of calibrant compounds added so that the calibrant peaks and the analyte peaks are simultaneously observed with reasonable intensity.

MALDI mass spectra may also be calibrated externally; this approach is experimentally simpler. In this method calibrant compounds are measured using the same matrix and laser irradiance as were used for sample analysis. A mass accuracy of ~0.1 to 0.2% (e.g., ±1 to 2 Da at m/z = 1000) can be achieved in linear mode using external calibration (Karas et al., 1989). In reflector mode, one can routinely expect mass accuracies on the order of 0.01% to 0.05% using external calibrations. In reflector mode it is also possible to use either the $[M+H]^+$ ion or the $[2M+H]^+$ ion (the protonated gas-phase dimer) of the matrix as one of the two calibrant peaks, which means that only one calibrant compound needs to be added.

MALDI Post-Source Decay MS for Peptide Sequencing

A peptide molecular ion that is sufficiently stable to be transported out of the ion source but insufficiently stable to survive the flight to the detector will decompose in the flight tube, giving rise to a series of fragment ions that are characteristic of the molecule's original structure. This process is referred to as metastable decay or post-source decay. It is believed that molecular ions acquire excess internal energy necessary for fragmentation via multiple collisions with matrix ions in the source (Kaufmann et al., 1994). When a precursor ion decomposes in the flight tube, all of the fragment ions will have the same velocity as the precursor, but they will have only a fraction of its kinetic energy. This means that the precursor and metastable fragments will all strike the linear detector at the same time and will be detected at the same apparent mass. Thus metastable or post-source-decay fragment ions are never observed in a linear spectrum.

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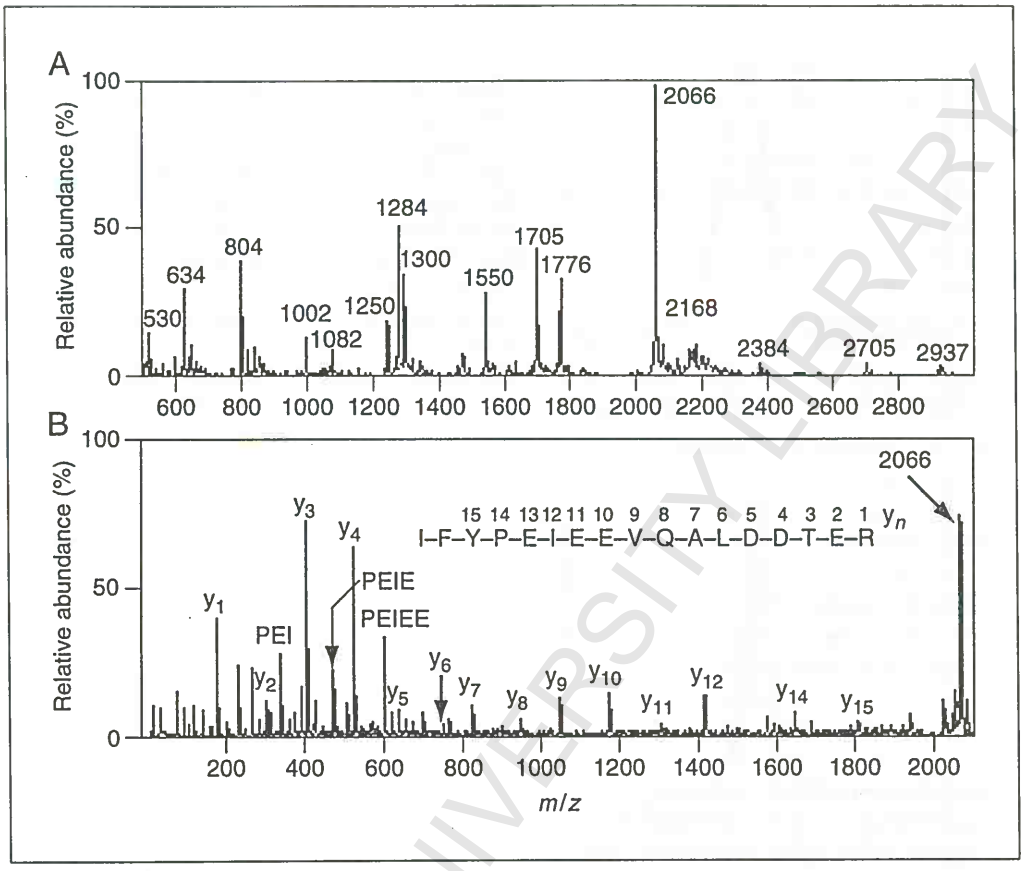

Figure 16.1.4 MALDI-MS analysis of a tryptic digest of an 18-kDa protein (Ladner et al., 1996). A total of 30 pmol of protein was separated on an SDS-PAGE gel, the band of interest excised from the gel, and the protein digested in situ with trypsin; then 1/20 of the sample was analyzed by MALDI-MS. (**A**) MALDI mass spectrum of the tryptic peptides recorded in the linear mode; (**B**) MALDI-PSD spectrum of the $[M+H]^+$ ion at *m/z* 2066. The peak was selected using an ion gate (R = 100). A nearly complete series of y_n ions (see Fig. 16.1.1) makes it possible to deduce the sequence of residues 3 to 17.

In contrast, a reflector TOF is capable of discriminating fragment ions by flight-time dispersion. Although all ions will have essentially the same velocity, the kinetic energy of a post-source-decay fragment ion will be determined by the ratio of its mass to the mass of the precursor. Thus the fragments, which have less kinetic energy than the precursor, will not penetrate as deeply into the ion mirror, and so will have their flight times decreased relative to the precursor. The fragments are therefore detected at a lower mass. By successively lowering the reflector voltage, thereby bringing lower- and lower-mass fragment ions into focus, a complete post-source decay fragment ion spectrum can be acquired in segments. The individual segments are calibrated and assembled afterward automatically by the data system.

The energy-focusing features of reflectron-type mass spectrometers make it possible to use post-source decay for peptide sequencing (Kaufmann et al., 1994). Post-source decay spectra resemble low-energy collision-induced dissociation (CID) spectra, similar to those commonly recorded on triple-quadrupole mass spectrometers (Hunt et al., 1986). The spectra contain mainly b_n and y_n ions, often with very abundant internal fragments and immonium ions (see Fig 16.1.1 and Table 16.1.2). The use of an ion gate makes it possible to perform a low-resolution (R = 100) selection of a precursor ion of interest from an unfractionated mixture, such as a protein digest. This approach is very powerful for providing internal sequence information on in situ–digested proteins that have been purified by SDS-PAGE (see Fig. 16.1.4 for an example).

ESI-MS

In electrospray ionization (ESI)-MS, ions are formed from peptides and proteins by spraying a dilute solution of these analytes at atmospheric pressure from the tip of a fine metal capillary (Fig. 16.1.5). The spray pro-

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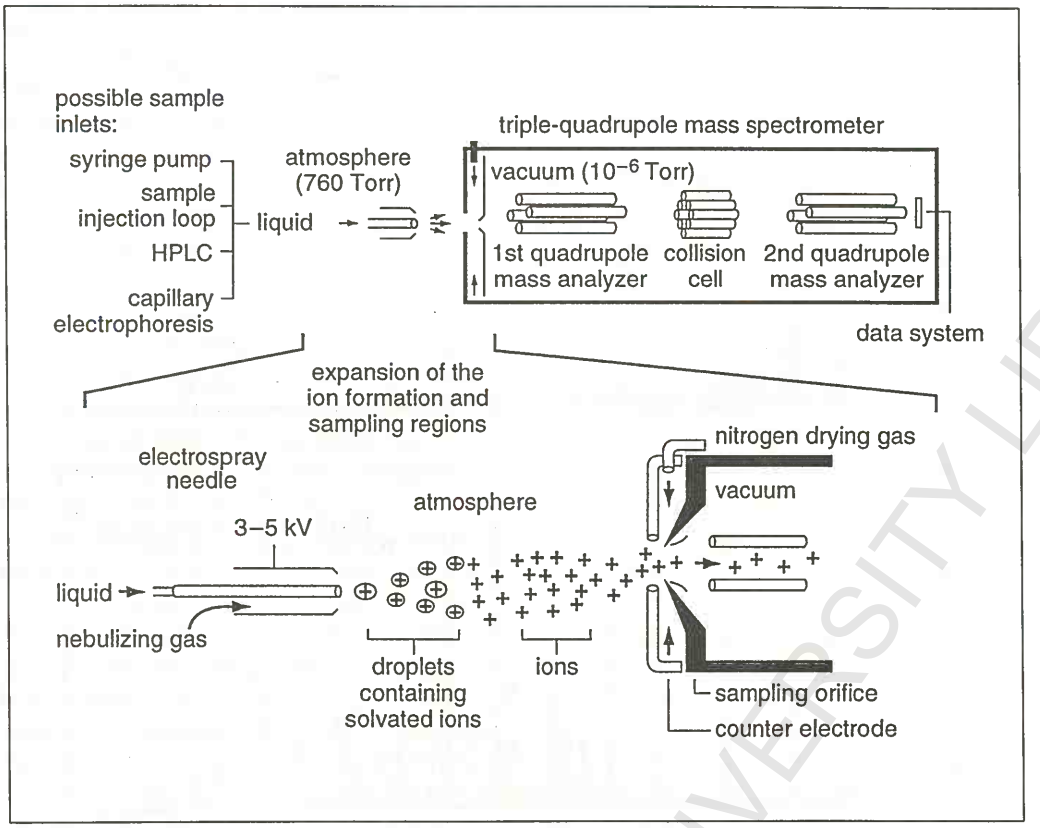

Figure 16.1.5 Schematic diagram of a triple-quadrupole mass spectrometer (top) and the electrospray interface (bottom).

cess, often assisted by pneumatic nebulization, creates a fine mist of droplets (Fenn et al., 1990; Kebarle and Tang, 1993). The droplets are formed in a very high electric field created by applying a high voltage (~4 kV) to either the spray tip (Fig. 16.1.5) or the counter electrode; in this process, the droplets become highly charged. Solvent evaporation is rapid from these small droplets. As the droplets evaporate, the peptide and protein molecules in the droplets pick up one, two, or more protons from the solvent to form singly or, more frequently, multiply charged ions (e.g., $[M+H]^{1+}$, $[M+2H]^{2+}$, etc.). The number of charges acquired by a molecule is roughly equivalent to the number of possible sites of proton attachment. As the droplets continue to shrink, the charge density on the surface of each one increases to the point where charge repulsion overcomes the forces holding together the droplet and the solvated ions contained within it. Ions are then "emitted" or "evaporated" from the droplet surface. The ions are sampled into the high-vacuum region of the mass spectrometer for mass analysis and detection, most often using a quadrupole (or triple-quadrupole) mass analyzer (Fig. 16.1.5). Usually little or no fragmentation is observed in the normal ESI mass spectra of peptides. The typical solvent for peptides and proteins is a mixture of water, an organic modifier such as CH_3CN, and up to a few percent by volume of acetic, trifluoroacetic, or another volatile acid (the latter included to enhance ionization of sample constituents). Because the ions are produced at atmospheric pressure from flowing liquid streams, ESI is ideally suited for on-line coupling to high-performance liquid chromatography, making it possible to analyze mixtures of peptides and proteins rapidly (see below).

As mentioned above, a key feature of the electrospray process is the formation of multiply charged molecular species from analytes that contain more than one possible site of proton attachment (Fenn et al., 1990; Smith et al., 1991). Proteins usually exhibit a characteristic series of multiply charged ions (e.g., see Fig. 16.1.8). As one proton (i.e., one charge) is typically attached for each 1000 Da of molecular mass, the ion series for a protein, even a large protein, will fall in the *m/z* range of 800 to 3000. This makes it possible for simple instruments like quadrupole mass spectrometers to be used for mass analysis of ions produced by ESI. The molecular mass of the protein can easily be calculated using the observed

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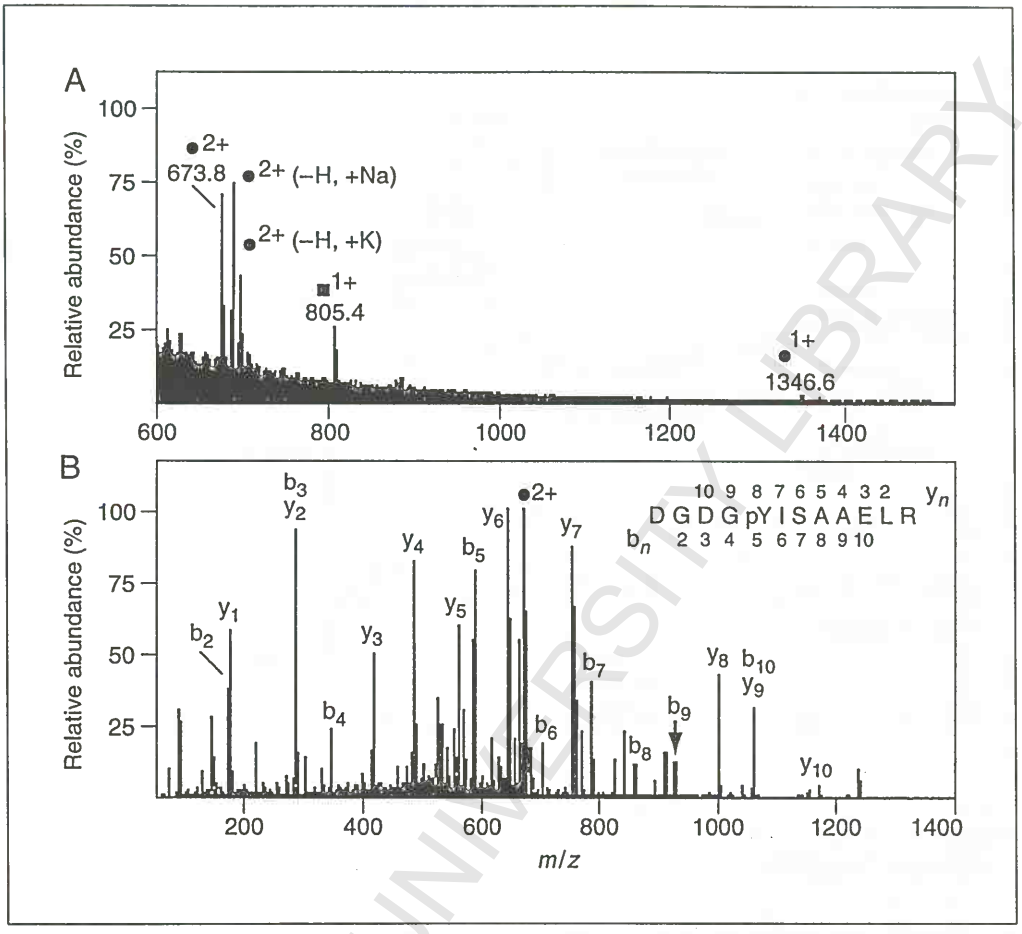

Figure 16.1.6 Electrospray analysis of an HPLC fraction from the tryptic digest of a 17-kDa phosphoprotein. The protein was purified by SDS-PAGE, electroblotted onto PVDF membrane, eluted, digested with trypsin, and the peptides analyzed by on-line LC-ESI-MS using a phosphopeptide-selective scanning method (Huddleston et al., 1993b). The column flow was split after UV detection, with 10% going to the mass spectrometer and 90% to a fraction collector. (**A**) Nanoelectrospray mass spectrum of a putative phosphopeptide-containing fraction. Peaks labeled (+Na) and (+K) correspond to $[M + H + Na]^{2+}$ and $[M + H + K]^{2+}$, respectively. (**B**) Collision-induced decomposition (CID) spectrum of the doubly charged ion ($[M+2H]^{2+}$) at $m/z = 673.8$. This spectrum confirms the sequence of the peptide and identifies the site of phosphorylation as Tyr^5.

masses of any two adjacent ions in the series (Covey et al., 1988; Mann et al., 1989; Reinhold and Reinhold, 1992). Normally these calculations are performed automatically by the data system. Because each multiply charged peak provides an independent measure of the molecular mass of the protein, an ion series from a single experiment provides a measure of the mass measurement precision. Most data systems also provide algorithms for transforming the mass-to-charge axis into a molecular mass axis for simplifying interpretation and presentation of the data. Proteins with molecular masses of up to 150,000 Da have been successfully analyzed by ESI using commercially available quadrupole instrumentation. Mass assignment accuracies of 0.01% are routine (see Table 16.1.1 and discussion of Fundamentals of Mass Measurement Accuracy and Mass Resolution). ESI has also been successfully interfaced with ion-trap mass spectrometers (Korner et al., 1996), Fourier-transform ion-cyclotron resonance mass spectrometers (Winger et al., 1993; O'Conner et al., 1995; Wu et al., 1995; Smith et al., 1996), and TOF mass spectrometers (Verentchikov et al., 1994; Mirgorodskaya et al., 1994).

In the case of peptides, the maximum number of charges observed often correlates reasonably well with the number of amino acid side chains that can readily accept a proton at the low pH of the analyte stream (i.e., Arg, Lys, and His, plus the free amino terminus). In the case of tryptic peptides, at least two charge sites are normally present, and tryptic peptides with molecular weights of 800 to 3000 typically

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exhibit a major, if not dominant, $[M+2H]^{2+}$ ion. For example, the ESI mass spectrum of an HPLC fraction from a tryptic digest of a phosphoprotein shows a simple mixture of peptides, in which doubly charged forms of the major peptide predominate (Fig. 16.1.6). In this spectrum, in addition to proton attachment, doubly charged ions due to Na and K adduction are also abundant. In general, the smaller the peptide, the more likely it is that the singly charged $[M+H]^+$ ion will also be observed. As the size of the peptide increases, triply and higher-charged states of the peptide will increase in abundance in the spectra while the abundance of ions with lower charge states will decrease (Fig. 16.1.7).

Electrospray mass spectra are keen reminders that mass spectrometers measure the ratio of mass to charge (*m/z*) and not molecular mass directly. The peak at *m/z* = 1346.6 in Figure 16.1.6 (panel A) is the monoisotopic $[M+H]^+$ of a peptide of molecular weight 1345.6 (see section on Fundamentals of Mass Measurement Accuracy and Mass Resolution for a discussion of monoisotopic mass). Evidence that this ion carries a single charge (1^+) as opposed to a higher number of charges is obtained from the spacing of the isotope peaks, which are one *m/z* unit apart. The doubly charged form of this same peptide is observed on the *m/z* scale at [1345.6 +2]/2 = 673.8. The isotope peaks in the $[M+2H]^{2+}$ isotope cluster are spaced 0.5 *m/z* units apart, indicating that these ions are doubly charged. Quadrupole mass analyzers can routinely achieve resolution sufficient to distinguish singly from doubly charged ions up to *m/z* = 3000 (if sufficient sample is available and the instrument is scanned slowly). This capability is particularly relevant to tryptic digests of proteins where, as noted above, the doubly charged ion may be the only ion observed for a given peptide. In the absence of the peak spacing information, the analyst could have difficulty determining if such an ion is singly or doubly charged. For ions with three or more charges, a charge series (e.g., 2^+ and 3^+) is usually present that permits the analyst to determine the charge state and mass of any given ion in the spectrum without the need to determine the isotopic peak spacing.

LC-ESI-MS

Electrospray ionization has made on-line liquid chromatography (LC)-MS a practical and robust method for sample introduction because gas-phase ions, as opposed to liquid droplets, are what is mainly being sampled by the instrument. The ideal milieu for presenting a sample to any mass spectrometer, regardless of the ionization mechanism, is in mixtures of highly pure water and volatile organic solvents containing low levels of volatile acid or base. Fortunately, such mixtures (e.g., water, acetonitrile, and trifluoroacetic acid at 0.05% to 0.1% by volume) are also those that provide optimal separation of peptides and proteins by reversed-phase liquid chromatography. From a practical standpoint, LC-MS reduces or eliminates the need to preparatively fractionate complex mixtures prior to MS, thereby saving valuable instrument time and preventing possible sample losses. LC-MS data may also be searched retrospectively for components present at very minor levels that may not have been considered worthy of collection based on a weak UV response.

In an LC-MS experiment, mass spectra are recorded continuously as the components elute from the HPLC column. If the mass spectrometer is made to scan once every 4 sec, the LC-MS data file will contain ~900 mass spectra. To identify regions of data that are likely to contain useful spectra, the mass spectrometer's data system produces a plot of the total number of ions detected during each mass spectrum scan versus time (the scan number). This plot, which is called a mass chromatogram or a total ion current (TIC) trace, looks very much like the UV chromatogram (Fig. 16.1.7). If it is of interest to know whether a particular peptide is present in the digest and where it elutes, the analyst simply requests the data system to display a selected ion-current trace for the specific masses of the most likely charge states (e.g., 1^+ to 4^+). Figure 16.1.7 illustrates the type of data typically acquired during the LC-ESI-MS analysis of a protein digest. In this example, the protein is heterogeneously glycosylated at a single site, and the multiplicity of peaks evident is due to differing carbohydrates attached to a single tryptic peptide (Fig. 16.1.7, panel C).

Because an ESI mass spectrometer behaves as a concentration-dependent detector, much like a UV detector, only a few percent of the total column effluent (~1 to 5 µl/min) needs to be directed into the mass spectrometer. The remainder can be diverted, via a flow splitter, through an in-line UV detector to a fraction collector. This arrangement makes it possible to perform straightforward correlation of the UV trace with the total ion current trace and then analyze individual fractions further by MS/MS (see discussion of ESI MS/MS for Peptide Sequencing) or some other method.

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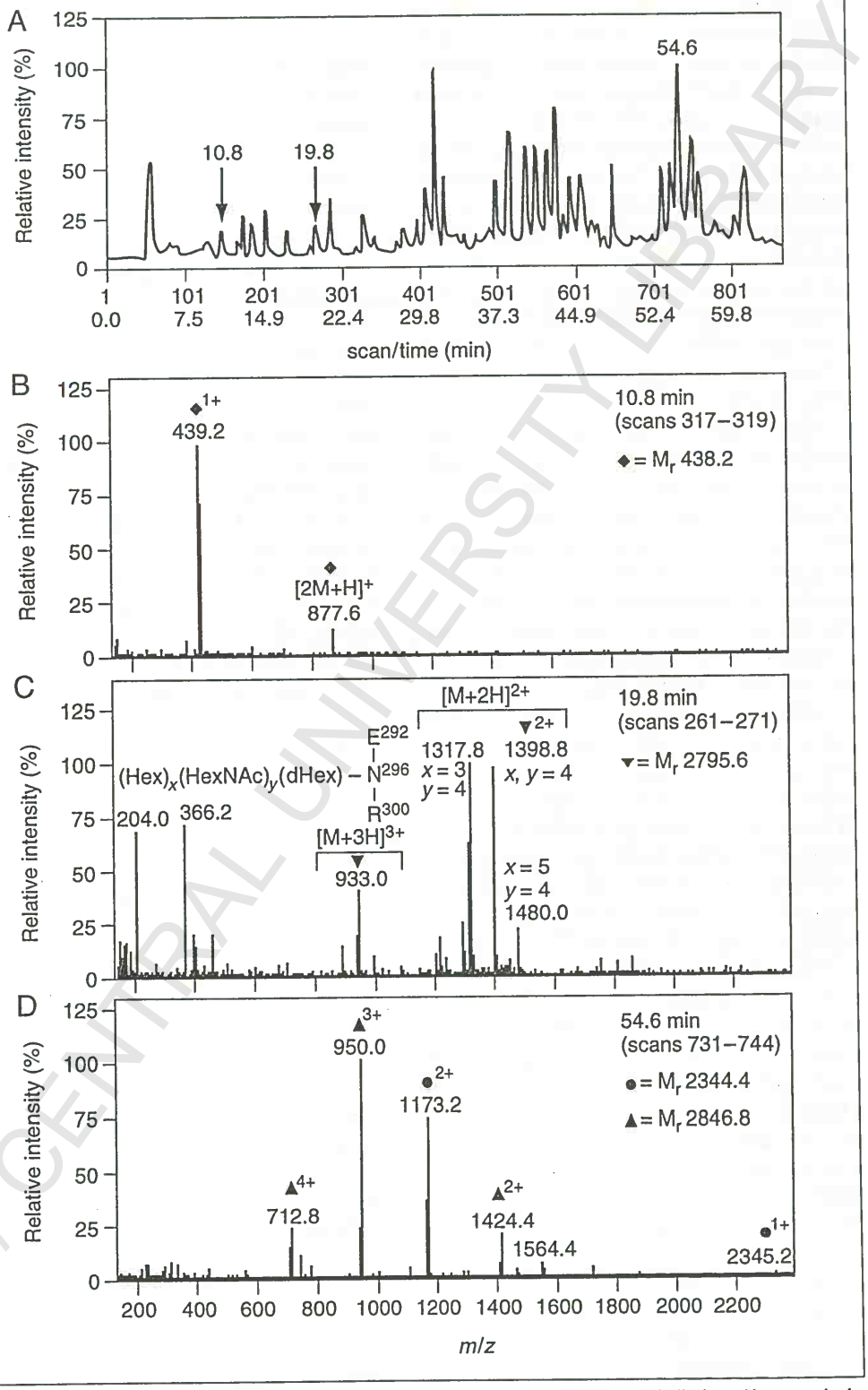

Figure 16.1.7 On-line LC-ESI-MS analysis of a tryptic digest of reduced and alkylated heavy chain (51 kDa) from a recombinant monoclonal antibody. (**A**) Total ion current trace showing the distribution of tryptic peptides. (**B**) ESI spectrum of the peak at 10.8 min showing a small singly charged peptide and its gas-phase dimer. (**C**) ESI spectrum of the peak at 19.8 min corresponding to a tryptic glycopeptide with heterogenous carbohydrate. At least five different carbohydrate structures are indicated. Abundant low-*m/z* ions at *m/z* = 204 and *m/z* = 366 are characteristic fragment ions produced from the carbohydrate (Roberts et al., 1995). (**D**) ESI spectrum of the peak at 54.6 min showing two large tryptic peptides, each represented by several different charge states.

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The UV response is also useful for estimating the amount of a peptide that has been injected on the column. This is generally not possible to do by MS without appropriate internal standards (see section entitled Is MS Data Quantitative?). LC-ESI-MS has become a key analytical method for the analysis of natural and recombinant proteins, and data from these experiments is now often included in the regulatory filings for new biopharmaceutical products (Carr et al., 1989; Palczewski et al., 1994; Watts et al., 1994; Stults et al., 1994; Rush et al., 1995; Schindler et al., 1995; Roberts et al., 1995; Resing et al., 1995; Hemling et al., 1996).

ESI MS/MS for Peptide Sequencing

The principles of MS/MS using triple-quadrupole mass spectrometers have been reviewed (Yost and Boyd, 1990). Briefly, the first quadrupole (Fig. 16.1.5) acts as a mass filter to select specifically the $[M+nH]^{n+}$ ion (where n is typically 1, 2, 3, or 4) of a peptide of interest and isolate it from other ions produced in the source. The selected precursor ion is then fragmented with a neutral gas such as nitrogen or argon in a quadrupole collision cell. This process is known as collision-induced decomposition. The fragment or product ions produced are then transmitted into the quadrupole second mass analyzer, which is being used as a scanning mass analyzer. The product ions are separated based on *m/z* and detected. The result is a product-ion mass spectrum of the selected precursor that is free of interferences from other components present in the mixture. An example of this process is shown in Figure 16.1.6. The fragmentation of this peptide reveals its sequence and the location of the phosphorylated residue.

Fragmentation can also be induced in the ESI source by increasing the energetics of the ion sampling process. This is done by adjusting the lens potentials in the high-pressure region upstream from the first quadrupole mass filter. Ions with increased kinetic energy undergo decomposition via collisions with residual gas molecules (Katta et al., 1991). Structurally useful fragmentation can thus be obtained on a single-quadrupole instrument, with the caveat that because this method has no ability to preferentially select precursor ions, it requires a highly purified sample. Recently strategies have been developed that take advantage of this ability of the ESI interface to generate fragment ions prior to mass analysis. Selective detection of glycosylated peptides (Carr et al., 1993; Huddleston et al., 1993a), phosphorylated peptides (Huddleston et al., 1993b; Till et al., 1994), and sulfated peptides (Bean et al., 1995) during on-line LC-ESI-MS analysis of protein digests can be accomplished by monitoring the total ion current trace for highly diagnostic low-*m/z* fragment ions produced in the ESI interface. These procedures are equally applicable to the detection of these modifications in unseparated mixtures.

IS MS DATA QUANTITATIVE?

A question that frequently arises in the analysis of peptide or protein mixtures is whether the relative peak heights (or areas) are quantitatively representative of the relative solution concentrations of the sample components. Unfortunately, the answer to this question is no, unless appropriate internal reference standards are used. Due to differing ionization and desorption efficiencies, the absolute yields of different ions from one peptide in a mass spectrometer can vary by perhaps as much as 2 orders of magnitude, depending on the sequence, composition, and size of the peptides (Jesperson et al., 1995; Dunayevskiy et al., 1995; Cohen and Chait, 1996). Furthermore, strongly ionizing components can frequently suppress the ionization of more weakly ionizing components present in the same sample or chromatographic fraction. In MALDI, the matrix itself can strongly influence the detectability of specific sample constituents (see discussion in *UNIT 16.2*). Quantitation by mass spectrometry requires that the response of a specific sample component be determined relative to that of a reference compound added to the sample and analyzed under identical conditions. In certain cases, semiquantitative estimates of relative amounts of components may be made if the components are of nearly identical structure and their chemical differences do not significantly affect the overall charge or hydrophobic/hydrophilic character of the molecules. For example, the relative ratios of the variants of β-casein observed in the ESI mass spectrum (Fig. 16.1.8) are probably a good reflection of the relative solution concentrations of these variants, as they differ from one another by only one or two amino acid substitutions out of more than 200 amino acids. The smaller the molecule, the more likely it is that such estimates will be in error.

SAMPLE PREPARATION

The ideal method for preparing samples for any type of MS analysis is reversed-phase high-performance liquid chromatography (RP-

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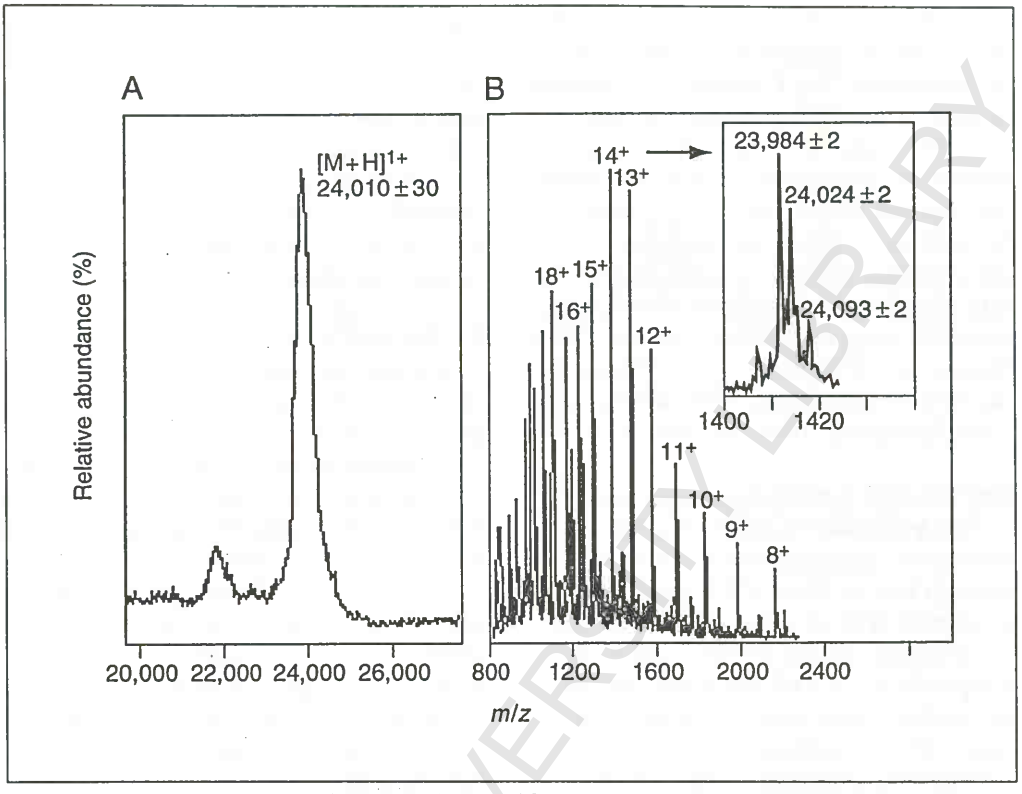

Figure 16.1.8 Molecular weight determination of β-casein, a 24-kDa phosphoprotein with known sequence heterogeneity. (**A**) Linear MALDI-TOF spectrum. Molecular weight was determined from the centroid of the smoothed peak using an external calibration. (**B**) ESI spectrum. The inset, an expansion of the region around the 14^+ charge state, reveals the presence of at least three variants. The molecular weights shown are the averages of determinations made from ten individual charge states.

HPLC). If purification by RP-HPLC is not an option because of known problems with sample loss, then the cleanup protocol employed should produce a sample composition that resembles as closely as possible that of a sample which has been purified by RP-HPLC. That is, the sample should contain the least amount possible of buffers, salts, detergents, and anything else besides water, organic modifier, and volatile acid or base.

The quality of the data obtained using ESI-MS is very dependent on the type and concentration of excipients present in the sample. If buffers are necessary for protein solubility or enzymatic digestion, it is best to use a volatile buffer such as ammonium bicarbonate or ammonium acetate at a concentration of ≤30 mM. In general it is best to avoid using any ionic detergents (surfactants), even though it is possible to obtain spectra for some proteins when these are included as long as the surfactant concentration is kept <0.01%. ESI tolerates nonionic detergents better. Analytically useful data may be obtained for sample solutions containing certain nonionic detergents at concentrations between 0.01% and 0.1% (Ogorzalek Loo et al., 1994).

Samples containing unacceptable amounts of buffers, salts, and detergents need to be purified prior to ESI-MS analysis. If the sample can be eluted from an RP column, some type of on-line procedure is both rapid and efficient. For water-soluble salts and buffers, a short, small-i.d. RP cartridge installed in place of the sample loop on a HPLC injector serves to both concentrate and purify samples (Stoney and Nugent, 1995). By placing the trapping cartridge in the injector loop, it is possible to wash the buffers and salts into waste rather than into the mass spectrometer. After loading the aqueous sample onto the trap (sample volume is no longer important except for very hydrophilic sample components), the trap is washed with 1% to 5% organic solvent and the salts and buffers are diverted to the waste line. The trap column is then is put in-line with the solvent delivery pump by switching the valve, and the sample is eluted into the mass spectrometer with the appropriate solvent. This is also a

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convenient way to analyze dilute solutions, as the trap serves to preconcentrate the sample.

The same approach can be used to purify samples containing anionic and nonionic detergents (Stoney and Nugent, 1995). However, on-line use of detergent columns require that this step be done in tandem with an RP column, because either the sample will have to be eluted with a buffer and thus will need to be desalted prior to introduction into the mass spectrometer, or it will not be retained on the column and will need to be trapped on an RP column. An alternative approach for removing SDS from samples is to load the sample, dissolved in a high concentration of organic solvent, onto a polyhydroxyethyl aspartamide column (Jeno et al., 1993). Under these conditions, peptides and proteins are retained while the SDS passes through with the solvent front. The sample is then eluted using a gradient of decreasing organic strength.

All of the purification strategies described above can, of course, also be performed off-line. In either case, the use of small-bore HPLC columns and a low-flow HPLC system will provide better results with sample amounts in the picomole range or below. Because the ESI mass spectrometer acts as a concentration-dependent detector, the highest sensitivity will be achieved using the smallest-i.d. columns. For off-line cleanup, the use of the lower flow rates required for small-bore columns means that samples are collected in smaller volumes. This can be important for small amounts of sample, where losses associated with reducing solvent volumes prior to the next step of analysis often are great. The authors have found that 0.5- to 1-mm-i.d. columns provide a practical compromise between on-line sensitivity and easy collection of fractions using a post-column flow splitter. A number of groups have developed sample purification and fraction collection methods that use packed fused silica capillary columns and solvent flow rates of 0.1 to 5.0 µl/min (Zhang et al., 1995; Gulden et al., 1996) or micropipets filled with packing material (Houthaeve, et al., 1995).

Other approaches to protein purification for MS include ultrafiltration (*UNIT 4.4*), TCA precipitation, size exclusion chromatography (*UNIT 8.3*), and microdialysis (*UNIT 4.4*). Among these alternatives, the authors find the easiest and most reliable methods for desalting samples or effecting a buffer change to be ultrafiltration using membranes of varying molecular weight cutoff (MWCO), and small-column (1-mm-i.d.) size-exclusion chromatography. Final volumes of 25 µl can be achieved using the ultrafiltration devices.

If the protein is being purified for subsequent proteolytic digestion with the ultimate goal being mass spectrometry–based peptide mapping, a suitable approach is SDS-PAGE followed by in situ enzymatic digestion (*UNIT 11.3*; Williams and Stone, 1995; Wilm et al., 1996; Schevchenko et al., 1996). The broad applicability and high resolving power of SDS-PAGE applied to protein mixtures makes this approach very useful for achieving difficult separations or for working with proteins that have poor solubility characteristics. Electrophoresing as little as a few micrograms of individual protein on an analytical gel will usually provide an informative peptide map. Following SDS-PAGE, the protein can also be blotted onto a membrane and enzymatically digested (*UNIT 11.2*; Patterson, 1995; Hess et al., 1993). Recovery of peptides from individual proteins appears to be similar regardless of whether they were produced from an in-gel digest or digestion off a membrane. If the peptide map is to be recorded using MALDI, the peptides can be sampled either directly from the digest or from the peptide extract. If the map is to be recorded using ES, then on-line LC-MS is usually the best approach.

Although MALDI is very much more tolerant of the presence of salts, buffers, and some detergents, it too generally benefits from having the sample in the purest form possible, especially when dealing with subpicomole amounts of proteins or low femtomole amounts of peptides. All of the methods discussed above work equally well for preparing samples for MALDI-MS analysis.

FUNDAMENTALS OF MASS MEASUREMENT ACCURACY AND MASS RESOLUTION

Monoisotopic Mass and Average Mass

Most of the elements that comprise typical biological molecules have more than one naturally occurring isotope, each with a unique mass and natural abundance. For example, carbon has two principle isotopes, ^{12}C and ^{13}C (see Table 16.1.3). The lighter, more abundant isotope of carbon has, by definition, a mass of 12.000000 and a natural abundance of 98.9%, while the heavier isotope has a mass of 13.003355 and a natural abundance of 1.1%. In a mass spectrometer one measures a large, statistical sampling of the molecules present and, therefore, observes all of the isotopes for

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Table 16.1.3 Isotopic Mass and Abundance Values

Isotope	Mass[a]	Natural abundance (%)[b]
^{1}H	1.007 825 035	99.985
^{2}H	2.014 101 779	0.015
^{12}C	12.000 000 000	98.90
^{13}C	13.003 354 826	1.10
^{14}N	14.003 074 002	99.634
^{15}N	15.000 108 97	0.366
^{16}O	15.994 914 63	99.762
^{17}O	16.999 131 2	0.038
^{18}O	17.999 160 3	0.200
^{19}F	18.998 403 22	100
^{23}Na	22.989 767 7	100
^{28}Si	27.976 927 1	92.23
^{29}Si	28.976 494 9	4.67
^{30}Si	29.973 770 1	3.10
^{31}P	30.973 762 0	100
^{32}S	31.972 070 698	95.02
^{33}S	32.971 458 428	0.75
^{34}S	33.967 866 650	4.21
^{36}S	35.967 080 620	0.02
^{35}Cl	34.968 852 728	75.77
^{37}Cl	36.965 902 619	24.23
^{39}K	38.963 707 4	93.2581
^{40}K	39.963 999 2	0.0117
^{41}K	40.961 825 4	6.7302
^{79}Br	78.918 336 1	50.69
^{81}Br	80.916 289	49.31
^{127}I	126.904 473	100

[a]Data from Wapstra and Audi (1985). Standard errors are given in this article but are omitted from the present table.

[b]Lide (1995).

each element present. The **monoisotopic mass** of a molecule is the sum of the accurate masses (including the decimal component, referred to as the mass defect) for the most abundant isotope of each element present (Yergey et al., 1983). As the number of atoms of any given element increases, the percentage of the population of molecules having one or more atoms of a heavier isotope of this element also increases. The most significant contributor to the isotopic peak pattern for biomolecules is the ^{13}C isotope of carbon. For peptides with molecular masses <2000 Da, the first peak in the resolved isotopic cluster corresponding to the all-^{12}C species will be the most abundant (Fig. 16.1.9, panel A). However, for peptides with molecular masses ≥2500 Da, the first peak in the isotopic cluster will no longer be the most abundant (Fig. 16.1.9, panel B). In this example, the species containing one ^{13}C atom is now the most abundant.

For proteins, the heavy isotopes of nitrogen, sulfur, and oxygen (in addition to carbon) make significant contributions to the mass, and, as a result, the monoisotopic peak in the molecular ion cluster appears to vanish (Fig. 16.1.9, panels C and D). In this case a different definition for the mass of the molecule must be used. Whenever it is not possible to observe the all-^{12}C peak for a molecular ion, the average mass should be used. The **average mass** of a molecule is the sum of the chemical average masses of the elements present (Fig. 16.1.9, peaks labeled A). The chemical average mass of an element is the sum of the abundance-weighted masses of all of its stable isotopes

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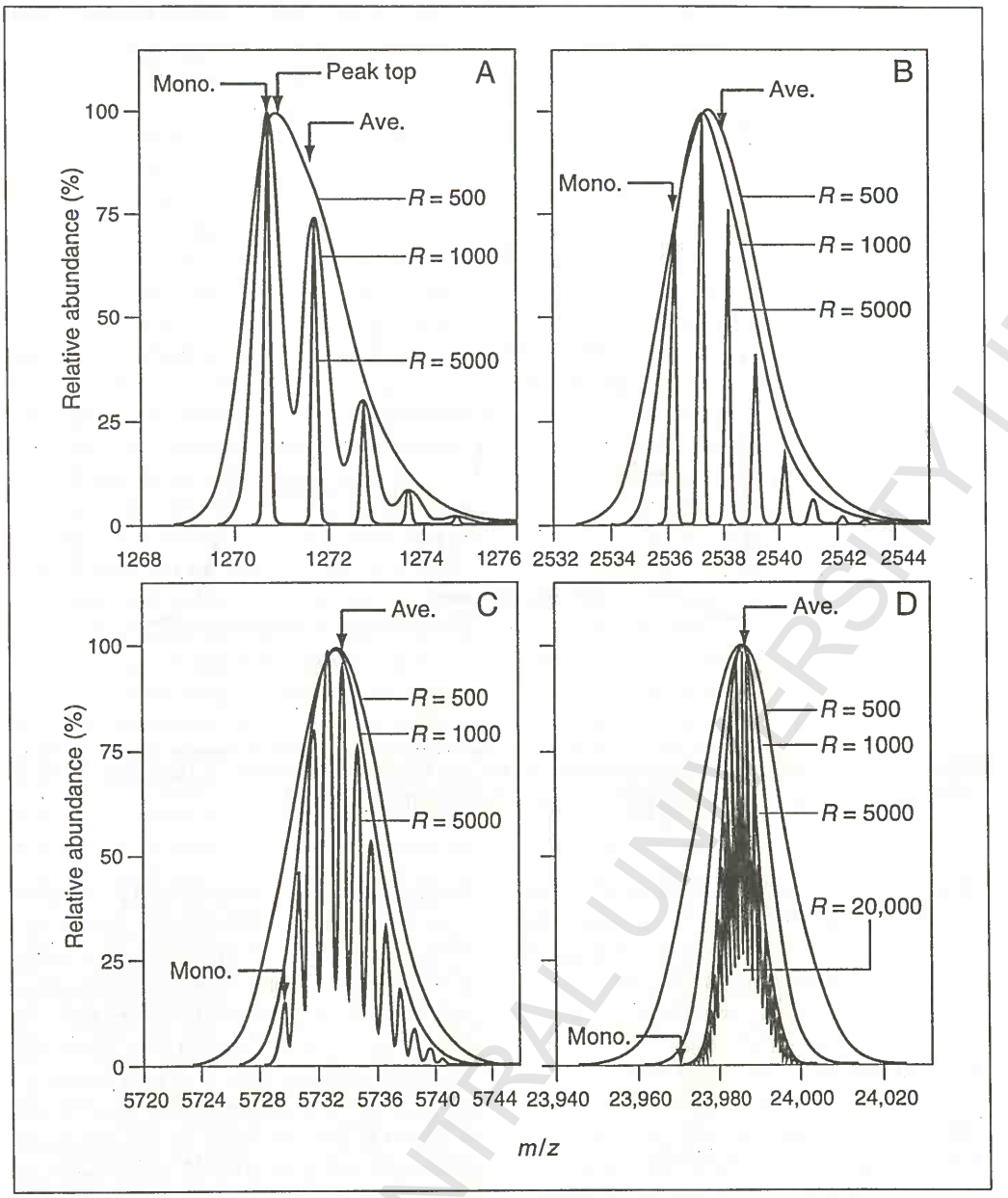

Figure 16.1.9 Effect of resolution (10% valley definition) on the appearance and relative peak intensity of the isotopic envelopes for representative peptides and proteins in four different mass ranges. Abbreviations: R, resolution; M, monoisotopic mass (Mono.); A, average mass (Ave.); P, peak top. **(A)** $C_{60}H_{86}N_{16}O_{15}$: M = 1270.7, A = 1271.5, P = 1270.8. **(B)** $C_{115}H_{161}N_{31}N_{35}$: M = 2536.2, A = 2537.7. **(C)** $C_{254}H_{377}N_{65}O_{75}S_6$: M = 5729.6, A = 5733.6. **(D)** $C_{1080}H_{1697}N_{268}0_{325}P_5S_6$: M = 23968.2, A = 23983.3.

(Yergey et al., 1983). For example, the isotope-weighted average mass of carbon is 12.0111, of hydrogen is 1.0079, of nitrogen is 14.0067, of oxygen is 15.9994, of sulfur is 32.0666, and of phosphorus is 30.9738. These masses are known with less precision than the monoisotopic masses of the elements because of variations in the natural abundances of the isotopes. The theoretical appearance of a molecule's isotope envelope may be precisely modeled by solving a polynomial expression that calculates the abundance-weighted sum of the isotopes of each element to the molecular ion cluster (Yergey, 1983). Nearly all modern mass spectrometer data systems come with programs that perform these calculations and can simulate the appearance of the resulting isotope pattern at different instrumental resolving powers.

Resolution

Whether or not one observes the individual isotopes that make up the molecular ion cluster

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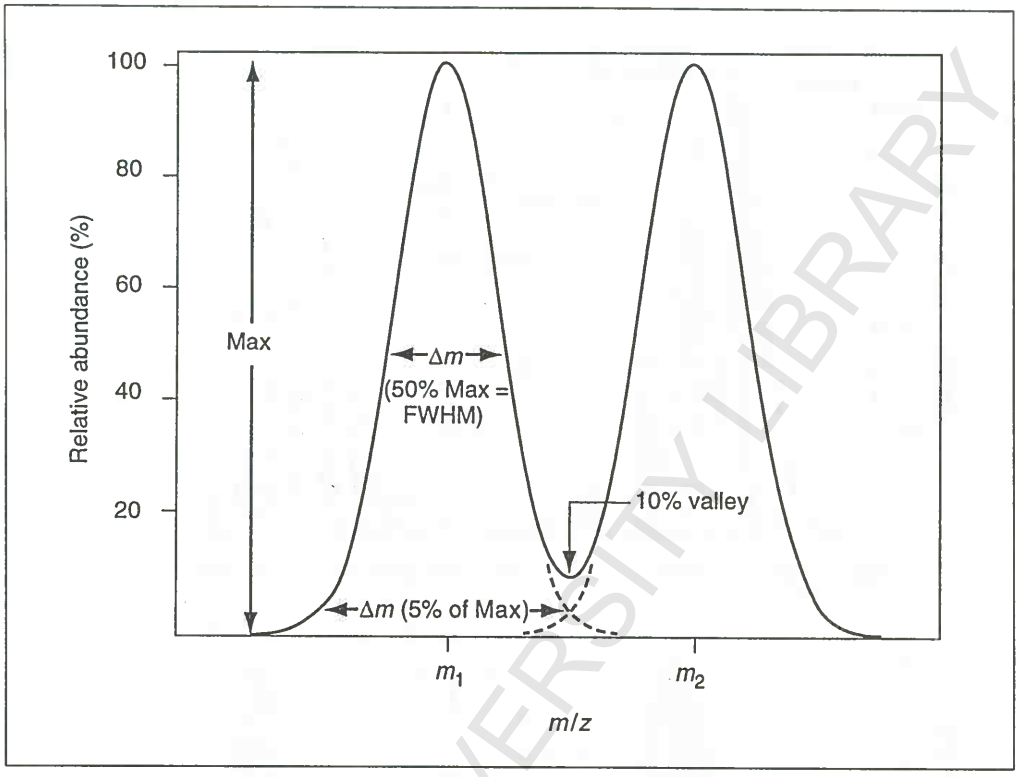

Figure 16.1.10 Schematic representation of the two common definitions of resolution used in mass spectrometry—10% valley and full-width, half-maximum (FWHM).

for a given compound depends on the mass resolution of the instrument being used. The resolution of the mass analyzer is a measure of its ability to separate signals of adjacent molecular mass. Mass resolution is often expressed as the ratio $m/\Delta m$, where m and $m+\Delta m$ are the masses (in atomic mass units or Da) of two adjacent peaks of approximately equal intensity in the mass spectrum (Fig. 16.1.10). There are presently two definitions of resolution in widespread use. In the "10% valley" definition, the two overlapping, adjacent peaks each contribute 5% to the resulting 10% valley between them (Fig. 16.1.10). The resolution of the mass analyzer may also be determined using a single peak by measuring the peak width (in m/z) at the 5% level and dividing this number into the m/z of the peak. The 10% valley definition is most frequently used for molecules with masses <5000 Da, where it is possible on certain analyzers to resolve the isotope peaks. For *unresolved* isotopic clusters the "full-width, half-maximum" (FWHM) definition is used. By this definition, the resolution is equal to the mass of the peak (in m/z) divided by the width (in m/z) of the isotopic envelope measured at the half-height of the peak (Fig. 16.1.10). An unfortunate consequence of the use of this definition is that, given an FWHM resolving value, it is more difficult to visualize what the separation between two peaks of a given masses will be. For example, a resolution of 1000 by the FWHM definition means that a peak at mass 1001 will be unresolved from the peak at mass 1000. Creation of a detectable separation between these two peaks would require a FWHM resolution >1200. A useful rule of thumb is that the value for the resolution determined using the FWHM definition is approximately twice that obtained using the 10% valley definition. For example, a resolution of 1000 using the 10% valley definition is approximately equivalent to a resolution of 2000 using the FWHM definition (i.e., the measured degree of separation of the peaks in Da/z is identical).

Mass Accuracy

Getting as accurate a molecular weight as possible is, of course, a central goal of any MS experiment. Here mass accuracy is taken to mean the difference in mass between the experimentally measured value and the theoretical value. By definition, the mass measurement accuracy can be directly determined only for compounds of known elemental composition and, therefore, known molecular mass. For unknown samples, the accuracy of the mass

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determination is approximated by measuring the mass of a peptide or protein of known elemental composition and similar mass to the unknown. It is important that these measurements be made under experimental conditions as close as possible to those used for analysis of the unknown. How useful this estimate of the mass measurement accuracy is depends on how well the instrument has been calibrated and on the reproducibility of the measurement. The reproducibility, or precision, of the measurement is best evaluated by making replicate measurements of the sample. However, in cases where the amount of sample is limited or the data is too noisy, a reasonable approach is to estimate the precision from replicate measurements of a known peak of mass and intensity similar to the unknown. It cannot be overemphasized that the mass spectrometer's performance with respect to mass measurement accuracy and precision must be regularly evaluated using well-characterized standards covering the mass range of interest in order to provide confidence in the measurements of unknowns being made. It is desirable to achieve a mass measurement precision of ±0.5 Da or less for peptides and ±1 Da for proteins. In practice, it is relatively easy to achieve the stated value for peptides but more difficult for proteins (Table 16.1.1). Whether the monoisotopic mass or the average mass should be used when measuring and reporting molecular weights will depend on the mass of the substance and the resolving power of the mass spectrometer (see discussion of Interplay Between Resolution and Molecular Weight).

The accuracy of the mass measurement may be stated in several different ways: as either an absolute mass error or a percentage of the measured mass (e.g., molecular mass = 1000 ±0.1 Da or ±0.01%), or as parts per million (e.g., molecular mass = 1000 ±100 ppm). As the mass being considered increases, the absolute mass error corresponding to the percent or ppm error will also increase proportionally (e.g., 0.01% or 100 ppm = 0.1 Da at m/z = 1000, 0.5 Da at m/z = 5000, or 5 Da at m/z = 50,000). It is important not to confuse mass accuracy with the term "accurate mass"; the latter is reserved for mass measurements accurate to ≤10 ppm.

Interplay Between Resolution and Molecular Weight

In the mass range up to ~3000 Da it is recommended that, if possible, a resolution sufficient to partially resolve the isotope cluster be used to facilitate mass assignment of the monoisotopic peak (Ingendoh et al., 1994). Resolution sufficient to separate single-mass-unit differences is also important in order to detect the presence of modifications such as deamidation of Asn to Asp and to make it possible to directly determine the charge state of an ESI ion by measuring the isotope peak spacing. For poorly resolved or unresolved isotope clusters, the mass accuracy can be dramatically affected by how the data is treated by the instrument's data system. It is important to be aware of whether the data sytem is assigning masses to the centroid of the peak or to the peak top. In general, the centroid and the peak top mass will not be the same in this mass range because the isotopic distribution comprising the unresolved envelope of peaks is asymmetric in shape. Below mass ~3000 it is sometimes difficult to accurately determine the centroid, and therefore the average mass, of an unresolved isotope cluster. The reason for this difficulty is that the bottom of the cluster, which must be included to get an accurate determination of the centroid for peaks in this mass range, is often distorted or poorly defined due to sample background (or, in MALDI, matrix) noise and/or poor ion statistics. These factors can easily contribute errors of ≥1 Da to the mass determination for compounds in the mass range of 1000 to 3000 Da. Furthermore, it should be noted that the peak top mass, which is the most easily determined value, corresponds to neither the monosiotopic mass or the average mass in this mass range. At ~1200 and below, the mass at the peak top of the unresolved envelope will be closer to the monoisotopic mass, whereas at ~3000 Da and above, the peak top will be closer to the average mass. For these reasons the use of resolved isotopes in this mass range is clearly preferable.

Determining the monoisotopic mass of a protein is difficult or impossible due to the insignificant contribution that the monoisotopic peak makes to the isotopic envelope (Fig. 16.1.10, panel D). Furthermore, quadrupole and TOF mass analyzers cannot provide the resolving power necessary to separate the isotope peaks for molecules with masses in excess of ~8000 Da. Fortunately, at masses above ~10,000, the unresolved peak profiles are quite symmetrical and the mass at the peak top is nearly identical to the chemical average mass of the protein. For proteins, errors in measuring the centroid can be caused by the presence of unresolved adducts along with the protonated molecular ion (with, for example, Na^+, K^+, or

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photochemical adducts of MALDI matrices) or of other proteins of similar but not identical mass that are not resolved. The relative abundance of any of these adducts is unpredictable, and will shift the apparent centroid of the unresolved cluster to higher mass in an unpredictable fashion. In these cases, it will be impossible to obtain an accurate mass assignment unless the instrument resolving power can be increased or the contributing adduct ions can be removed by more stringent cleanup of the sample (Ingendoh et al., 1994; Zubarev et al., 1995).

Thus it is still important even at higher mass to have a reasonable amount of resolving power. Resolution should be sufficient to enable detection of common protein variants that may be present in the sample. For example, β-casein exists as a family of isoforms differing by the substitution of one or more amino acids. The MALDI and ESI mass spectra of β-casein are compared in Figure 16.1.8. Detection of one of these variants, which differs by 40 Da in 24,000 Da, requires a resolution of ~1200 FWHM ($24{,}000/40_{10\%\ \text{valley def.}} \times 2$), which is easily achievable by ESI-MS on quadrupole mass analyzers. The resolution in MALDI-MS with a TOF analyzer is typically <1000 at this mass, and is insufficient to detect such small modifications. The minimum desirable resolution can be estimated from the natural width of the molecular ion envelope obtained from the computer programs mentioned above. For example, the envelope of isotopes for a 24-kDa protein has a width of ~10 Da at the half-height of the peak (Fig. 16.1.9, panel D). This corresponds to an apparent resolution of 2400 (24,000/10). Having an *instrumental* resolution higher than 2400 but less than ~20,000 (see Fig. 16.1.9) will not improve the apparent resolution because the minimum width of the unresolved protein molecular ion cluster is determined by the weighted average of the natural abundance of the isotopes present. It should be clearly stated, however, that having a higher resolution than is theoretically required is not detrimental (as long as it does not result in a decrease in sensitivity), and it may have some beneficial effect on the ability to precisely define the peak top and therefore the average mass.

OUTLOOK

Mass spectrometry and its application to biological problems are clearly enjoying a second childhood. MS-based methods for protein sequencing have sensitivity in the femtomole range, exceeding that of Edman-based sequencing, and MS-based methods are uniquely capable of analyzing the ever-broadening spectrum of protein modifications. Mass spectrometry is even showing promise as a tool for studying protein folding (Englander, 1993; Smith and Zhang, 1994; Wagner and Anderegg, 1994) and noncovalent interaction (Smith and Light-Wahl, 1993; Cheng et al., 1995). Further increases in the understanding of the ionization/desorption processes in ESI and MALDI are likely to translate into even higher sensitivity through higher ion-sampling efficiency. It can also be expected that new methods for dissociating molecular ions of peptides and proteins (possibly involving surface-induced collisions or photoexcitation) will be developed that will be more efficient or more selective than collision-induced or post-souce decay. One can also anticipate that computer algorithms for the interpretation or "fingerprinting" of MS/MS data will continue to improve so that soon a minimum amount of human intervention will be required for identification (for an example, see Eng et al., 1994). Although MS-based approaches have sufficient raw sensitivity to analyze low femtomole amounts of peptides and proteins, purifying and handling samples at these levels remains a significant challenge for which there is no general strategy at present. With further improvements, gel- and membrane-based methods and microchromatography-based methods for immobilizing minute amounts of sample for biochemical manipulation and cleanup may meet this challenge.

The mantra for MS now and into the next millenium is "smaller, cheaper, faster, simpler, more sensitive." These criteria are based in large part on the need to make MS more accessible to the biological community. A variety of new types of mass spectrometers have recently been introduced that satisfy one or more of these criteria, and certain of these new instruments may provide unique benefits for molecular mass determination and sequencing of minute amounts of peptides and proteins. ESI and MALDI have shown particular promise when coupled to quadrupolar ion trap analyzers (Korner et al., 1996; Qin and Chait, 1995). These devices are capable of very high resolution and very rapid scanning, and can perform MS^n experiments, in which fragment ions formed in the first MS/MS (MS_2) experiment are further fragmented to provide additional structural information. TOF mass analyzers have been coupled to ESI sources (Verent-

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chikov et al., 1994; Mirgorodskaya, 1994) and offer the promise of improved resolution, very high sensitivity, and very fast sampling rates. An interesting variation on these two approaches couples an ion trap to a TOF analyzer, providing structural information as well as molecular mass data (Lee and Lubman, 1995). Additional variations on this theme of connecting two different, relatively inexpensive analyzers together to produce a more versatile instrument are under development. Although neither small or inexpensive, Fourier-transform ion-cyclotron resonance mass spectrometers equipped with MALDI and ESI are extremely versatile instruments with the potential for simultaneously providing very high mass accuracy, high resolution (routinely ≥20,000), and extremely high sensitivity for peptide and protein mass measurement (Winger et al., 1993; O'Conner et al., 1995; Wu et al., 1995; Smith et al., 1996). These devices are also capable of performing MS^n experiments.

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Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Analysis of Peptides

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS; Karas and Hillenkamp, 1988; Tanaka et al., 1988; Hillenkamp et al., 1991; Beavis, 1992; Bahr et al., 1994; Kaufmann, 1995; Stults, 1995) is one of the most useful techniques for determining the mass of biomolecules, with exceptional capabilities for mass analysis of peptides. Relative to other ionization techniques, MALDI provides high sensitivity and excellent tolerance of salt and other common buffer components. Routine detection limits for peptides are in the subpicomole range. The ions commonly observed are the protonated molecules ($M+H^+$), which makes data analysis relatively easy. The use of MALDI-MS is becoming widespread, due in part to the availability of modern, commercially available instruments that are easier to operate and provide greater performance at lower cost than earlier versions.

Peptide analysis is one of the most important applications of MALDI-MS. The ability to ionize many peptides in a mixture simultaneously makes MALDI-MS a popular choice for rapid analysis of protein digests. In fact, the high sensitivity and the high salt tolerance of MALDI have permitted the mass analysis of peptides directly from a single neuron without using purification (Li et al., 1994). MALDI-MS is frequently used for analysis of HPLC fractions from peptide maps. It has also been coupled with capillary electrophoresis (CE; see *UNIT 10.9*) for off-line analysis (Keough et al., 1992; vanVeelen et al., 1993; Walker et al., 1995). Many other specialized applications of MALDI-MS have been described in the literature, including protein identification by peptide mass fingerprinting (Henzel et al., 1993; Mann et al., 1993; Pappin et al., 1993; Mann, 1994; Patterson, 1995), C-terminal peptide sequencing (Patterson et al., 1995; Thiede et al., 1995; Woods et al., 1995), post-translational modification determinations (Huberty et al., 1993; Yip and Hutchens, 1993; Liao et al., 1994; Robertson et al., 1994; Stahl et al., 1994; Sutton et al., 1994; Talbo and Mann, 1994; Crimmins et al., 1995; Rüdiger et al., 1995), and protein epitope mapping (Papac et al., 1994; Zhao and Chait, 1994).

Another popular application for MALDI-MS of peptides is as a complement to Edman degradation (Elicone et al., 1994; Geromanos et al., 1994). A small aliquot (10 to 100 fmol) can be removed prior to Edman degradation to provide information on the number of cycles for which the sequencer should be programmed. The peptide mass can be used to confirm the sequence obtained by Edman degradation and may in some cases provide indirect information on a residue that was not observed in the sequence data. A difference in the mass calculated from the sequence and that measured by MALDI-MS may indicate the presence of a post-translational modification.

INSTRUMENT CONFIGURATIONS

Most often the MALDI ion source is mated to a time-of-flight (TOF) mass analyzer from which the high mass range, resolution, and mass accuracy characteristics are derived (Cotter, 1992). The basic TOF mass spectrometer is a linear instrument, where the ions follow a straight, field-free path between the acceleration of the ion source and the detector. This configuration provides the highest sensitivity, yields few, if any, fragment ions, and requires the least manipulation of tuning parameters. The limitations of this instrument include relatively poor resolving power ($m/\Delta m$ <2000) and poor mass accuracy (±0.15% with external calibration). Resolving power is the ability to distinguish peaks that are close in mass. For TOF instruments, it is defined by the mass of a peak divided by its width at half-height. Reflector instruments (or mode of operation) yield higher resolution ($m/\Delta m$ ~2000 to 5000) and better mass accuracy (±0.015% with external calibration). The reflectron is a focusing device that improves resolution by allowing ions of the same mass with different energies to arrive at the detector at the same time. The improved performance, however, is often obtained at the expense of slightly lower signal and the occasional appearance of undesired fragment ions. MALDI has been described as a "soft" ionization technique; however, significant fragmentation often occurs, but it is apparent only when operating in the reflector mode. Fragmentation is rarely observed in a linear TOF instrument because no electric field separates the fragments from their precursor ions. Although the fragment ions can be quite useful for sequence determination in post-source decay (PSD) spectra (Kaufmann et al., 1993, 1994; see *UNIT*

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16.1), the fragment ions in the full-energy reflector spectrum do not appear at the proper masses and caution should be exercised so that they are not mistaken for molecular ions. Acquisition of an accompanying spectrum in the linear mode is the easiest method to ensure the proper identity of ions.

Particularly when operating in reflector mode, it is necessary to determine whether the instrument reports average or monoisotopic peak values (see *UNIT 16.1*). Low-mass peptides (<1200 Da) display well-resolved isotope peaks that are assigned as monoisotopic peaks by most data systems. Higher-mass peaks (>2000 Da) in the same spectrum may show little or no isotope resolution and the data system will report the average mass value. Peaks that are intermediate in mass will have partially resolved isotope peaks, and the data system may report without an indication whether the mass is monoisotopic or average.

A relatively new configuration, "delayed extraction," delivers higher resolution by temporally uncoupling the ionization event from ion acceleration (Colby et al., 1994; Brown and Lennon, 1995; Vestal et al., 1995; Whittal and Li, 1995). It is useful in both linear ($m/\Delta m$ = 2000 to 4000) and reflector modes ($m/\Delta m$ = 3000 to 6000). When delayed extraction is used in the reflector mode, a smaller degree of fragmentation is observed; this allows operation at higher laser power and results in higher signal intensity of the molecular ion. The higher resolution and mass accuracy afforded by delayed extraction have helped to give TOF-MS more of the characteristics commonly expected of a "high performance" mass spectrometer.

SAMPLE PREPARATION GUIDELINES

Sample preparation is straightforward and rapid (Lecchi and Caprioli, 1996). It is generally identical for all types of MALDI instruments. Peptide samples, either single components or mixtures, are cocrystallized with a large excess (~10^4) of a UV-absorbing compound (the "matrix"). The peptide molecules are released into the gas phase and ionized by a pulse of UV radiation, typically from a nitrogen laser (λ = 337 nm). A wide range of peptide concentrations can be analyzed, from 10^{-4} M (100 pmol/µl) to 10^{-8} M (10 fmol/µl). Often simply adding the matrix to the sample on the sample stage will result in high-quality spectra, as described in *UNIT 11.6*. Samples may also be prepared by mixing the sample solution and the matrix solution in a microcentrifuge tube. A total of 0.5 to 2 µl is applied to the target.

A large number of different matrices have been tried for peptide analysis; however, only two matrices are in common use: α-cyano-4-hydroxycinnamic acid (αCN; Beavis et al., 1992) and 2,5-dihydroxybenzoic acid (DHB; Strupat et al., 1992). A third matrix, less frequently used, is 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA; Beavis and Chait, 1989), which has proven useful for high-mass peptides (see Fig. 16.2.1 for chemistries and some guidelines for choosing a matrix). For a peptide with mass >1000 Da, αCN is preferred; DHB is a cleaner matrix for peptides of lower mass, and it is more tolerant of buffer salts at very low peptide concentrations. None of the matrices gives rise to significant photoadduct peaks with peptides, although photoadducts are often observed with proteins. The matrices are prepared as 10 mg/ml solutions in the solvents listed. Short-cuts, such as preparation of saturated solutions in these solvents, are occasionally used but yield less reproducible results. The matrix solutions are stable for weeks if they are kept in the dark and refrigerated when not in use. The matrices are normally used as obtained, without further purification. These compounds are photoreactive, and bad lots of material may be obtained on occasion. In addition, some commercial preparations contain salt contamination, particularly sodium, that will produce salt adducts and reduce the limits of detection. To avoid this, the matrices may be recrystallized and desalted by ion-exchange chromatography (*UNIT 8.2*). Alternatively, highly purified matrix preparations are available from some instrument vendors.

The detection limit for a peptide is dependent on the peptide's ionization efficiency, its mass, its hydrophobicity, its solubility, the availability of a basic site for protonation, and other unknown factors. MALDI is relatively tolerant of salts and small amounts of some detergents when compared to most other forms of mass spectrometry ionization (Mock and Sutton, 1992; Rosinke et al., 1995; Cohen and Chait, 1996; Gharahdaghi et al., 1996). Table 16.2.1 lists salt and detergent concentrations that are compatible with MALDI. Some of these components interfere with ionization, and others can prevent crystal formation. As a general rule, any component present in a concentration higher than that of the matrix (~50 mM) may be a problem. Detection limits are quite dependent on sample purity, especially at

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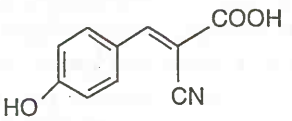
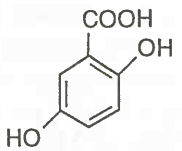
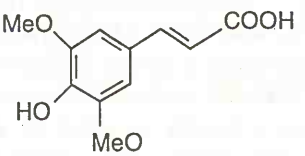
Compound name	Suggested solvent	Mass range (Da)
α-cyano-4-hydroxy-*trans*-cinnamic acid (αCN, CCA)	50% acetonitrile	400-100,000
		
2,5-dihydroxybenzoic acid (DHB, gentisic acid)	50% ethanol	200-5,000
		
3,5-dimethoxy-4-hydroxy-*trans*-cinnamic-acid (sinapinic acid, SA)	50% acetonitrile	1,000-100,000
		

Figure 16.2.1 Common matrix compounds for MALDI-MS.

Table 16.2.1 Tolerance for Common Buffer Components

Buffer component[a]	Maximum concentration[b]
Sodium chloride	50 mM
Phosphate	10 mM
Tris base	50 mM
Urea	1 M
Guanidine	1 M
Azide	0.1% (v/v)
Glycerol	1% (v/v)
PEG 2000	0.1% (w/v)
SDS	0.01% (w/v)
Triton X-100, RTX-100, or NP-40	0.1% (v/v)
Tween	0.1% (v/v)
CHAPS	0.01% (w/v)
n-Octyl-β-glucopyranoside	1% (v/v)
Zwittergent	0.1% (v/v)
Lauryldimethylamine oxide (LDAO)	1% (w/v)

[a]Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

[b]These values should be viewed as approximate. The maximum concentrations that can be tolerated depend on the matrix used, the method of sample preparation, the extent of rinsing, the amount of analyte, and the presence of mutiple buffer components.

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the subpicomole levels. The presence of buffer components and other peptides may lower the signal of the component of interest. In all cases, best results are obtained when salts and detergents are kept to as low a concentration as possible.

A number of methods have been developed that enhance the tolerance for buffer components. The easiest method is simple dilution; due to the excellent sensitivity of MALDI-MS, samples may be diluted significantly in order to reduce the buffer concentration. If the buffer is volatile, it may be removed by drying with vacuum centrifugation. Unfortunately, this approach often leads to sample losses by adsorption to the tube walls. A strong solvent (e.g., 70% isopropanol/5% trifluoroacetic acid) may assist in recovery of the sample. Often salts will crystallize separately from the matrix and analyte, so a simple, quick rinse with cold dilute acid is often useful with αCN or SA (Beavis and Chait, 1990); DHB is too water-soluble to permit rinsing. Rinsing the sample with water not only may enhance the signal for some peptides but also, in some cases, may result in detection of ions that are completely suppressed by the salt. However, care should be exercised because some hydrophilic peptides may be lost by washing. Matrix additives such as 50 mM fucose (Billeci and Stults, 1993) or 10% 5-methoxysalicylic acid (Karas et al., 1993) have been developed; in some case these additives can enhance the quality of spectra when buffer salts are present. Adsorption to nylon (Zaluzec et al., 1994), polyethylene (Blackledge and Alexander, 1995), nitrocellulose (Mock et al., 1992; Preston et al., 1993; Bai et al., 1995), Nafion (Bai et al., 1994), or polyvinylidene difluoride (PVDF; Vestling and Fenselau, 1994) allows extensive rinsing before matrix is added. In addition, adsorption to a membrane provides a platform for chemical or enzymatic reactions (Fabris et al., 1995). An excellent alternative for desalting a sample utilizes a short fused-silica capillary that contains a small volume of reversed-phase HPLC packing material (Zhang et al., 1995). A sample is concentrated on this capillary column, the salts are rinsed away, and the analyte is eluted directly onto the MALDI sample stage with 1 µl of organic mobile phase. Another alternative method is attachment of affinity molecules (e.g., antibodies) to the sample stage; this permits selective concentration of targeted analytes from dilute solution and allows for subsequent washing before the matrix is added to disrupt the affinity binding (Nelson et al., 1995).

Using higher laser power can increase detection of larger peptide fragments as well as detection of some peptides at very low levels. Some ions may not appear until higher laser power is used. Excessive laser power, however, can result in a loss of resolution, and in reflector-TOF operation, increased fragment ion formation may result in a loss of signal for the molecular ion. In general the laser power should be set to the lowest level that will still produce a good-quality signal.

Preparation of sample either in a microcentrifuge tube or on a sample stage with matrix is a rather straightforward task. Over recent years a number of alternative methods have been described. These include the thin-film method (Xiang and Beavis, 1994) and the rapid evaporation method (Vorm and Mann, 1994; Vorm et al., 1994). Advantages of these methods include easier rinsing of contaminants, lower detection limits, and/or more uniform crystal formation. A modification of the rapid evaporation method incorporates nitrocellulose for more reproducible results (Shevchenko et al., 1996). The exact method of sample preparation can have a significant effect on the results (Cohen and Chait, 1996), although no one method appears to be universally successful. These authors have tried these different techniques for many samples and find that similar results are often obtained. The lowest detection limits have been achieved when significantly smaller samples are used (<1 nl; Jespersen et al., 1994), but these require specialized sample-handling devices (picoliter vials).

INSTRUMENT CALIBRATION

The mass axis of the time-of-flight mass spectrometer is normally calibrated with a two-point calibration procedure. For peptides, these points are either a high- and a low-mass peptide or a single peptide plus a matrix ion for the low-mass point. The spectrum used for calibration should be acquired under the same conditions as the analyte spectra (same matrix, peptide concentration, and laser power). Table 16.2.2 is a list of common peptides used for calibration and their masses. Mass accuracy for external calibration is typically ±0.15% of the ion mass (e.g., ±1.5 Da at 1000 Da) for linear mode, and ±0.015% for reflector mode. Addition of mass standards to the analyte sample (internal calibration) can improve the accuracy to ±0.01%. The standards are chosen to bracket the analyte mass (above and below); they are added optimally at a concentration near that of the analyte. Peptides used for external calibra-

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Table 16.2.2 Common Mass Calibration Compounds

Sample name	M+H$^+$ (^{12}C)	M+H$^+$ (avg.)[a]
DHB matrix	155.0344	155.13
αCN matrix	190.0504	190.18
αCN matrix (dimer)	379.0930	379.35
desArg bradykinin	904.4681	905.04
Bradykinin	1060.5692	1061.23
Angiotensin I	1296.6853	1297.50
Substance P	1347.7360	1348.65
Glu-fibrinopeptide B	1570.6774	1571.60
Renin substrate	1758.9331	1760.04
ACTH-CLIP (18-39)	2465.1989	2466.71
Melittin	2846.7459	2848.49
Bovine insulin B-chain (oxidized)	3494.6513	3496.94
ACTH (7-38)	3657.9294	3660.17
Bovine insulin	5730.6087	5734.55
E. coli thioredoxin	—[b]	11676.44

[a]Due to variation in isotope abundance, the average mass is precise to only two decimal places.

[b]The nonisotopic peak abundance is too small to be observed.

tion (see Table 16.2.2) can also be used as internal calibration standards. The authors find that addition of internal standards for analyte levels <100 fmol has limited utility because the standard can suppress ionization of the analyte, and mass accuracy is compromised by poor ion statistics at low levels. A matrix peak may be used for a one-point calibration adjustment (Vorm and Mann, 1994).

ANALYSIS OF PEPTIDE MIXTURES

MALDI-MS is often the method of choice for rapid analysis of peptide mixtures. Ion suppression does occur in MALDI, but it is possible to analyze peptide mixtures containing dozens of components. Although the number of papers that describe applications of complex peptide mixtures is limited, spectra of unseparated tryptic digests have been published for human growth hormone and tissue plasminogen activator (Billeci and Stults, 1993), human interferon α and interleukin-4 (Tsarbopoulos et al., 1994), barley α-amylase (Andersen et al., 1994), and recombinase A (Lecchi and Caprioli, 1996). Complex peptide mixtures should, when possible, be analyzed using two different matrices. Each matrix may result in some differences in peak abundance (Lecchi and Caprioli, 1996). These differences may in some cases result in the appearance of new peptide ions and the disappearance of other ions. Measurements made using more than one analyte concentration may also lead to greater numbers of peptides being observed. Finally, spectra should be obtained from a number of different spots or crystals on the target because the homogeneity of the analyte can vary from crystal to crystal.

Detection of some ions may be limited by MALDI instruments with low resolution. For example, when a 100-kDa protein is digested with a protease such as trypsin, a large number of peptides with similar masses are typically generated. As the peptide masses approach one another (±1 to 2 Da), the ions may not be resolved. Instruments with higher resolution ($m/\Delta m$ >3000) will be able to separate masses that are within 1 Da. Larger peptides (>3000 Da) can be more problematic to analyze due to low ionization efficiency and low solubility. Peptides produced by cyanogen bromide are often hydrophobic and can be refractory toward MALDI analysis. Methods have been developed that provide some success by extraction of cyanogen bromide–generated peptides from nitrocellulose (Bai et al., 1995), cationic (Zhang et al., 1994), and PVDF membranes (Henzel et al., 1994) after electroblotting from SDS-PAGE. Octyl-β-glucoside (Cohen and

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Table 16.2.3 Autolytic Fragments of Trypsin and Lysine-C

Trypsin (porcine)		Lysine-C (*Achromobacter*)	
Peptide	Avg. mass[a]	Peptide	Avg. mass[a]
T2	261.3	K2	1922.1
T3	514.6	**K1**	3094.5
T7	842.0	**K5**	4875.3
T13	906.1	**K4**	5503.5
T10	1006.1	K3	5917.5
T6	1045.2	K6	6514.0
T9	1469.7		
T14	1737.0		
T8	1769.0		
T11	2158.5		
T4	2211.4		
T5	2283.6		
T12	3014.3		
T1	4491.1		

[a]Masses in bold print are the most frequently observed fragments. All masses are isotopic averages.

Chait, 1996; Gharahdaghi et al., 1996) has been a useful additive to increase the solubility of hydrophobic peptides.

Peptides can be generated in solution (*UNIT 11.1*), from in situ digestion on a PVDF or nitrocellulose membrane after electroblotting from a gel (*UNIT 11.2*), in an SDS-polyacrylamide gel (*UNIT 11.3*), in a silver-stained SDS-polyacrylamide gel (Shevchenko et al., 1996), or directly on the sample stage of a mass spectrometer (Fabris et al., 1995; Nelson et al., 1995). Most of these methods are directly compatible with MALDI analysis, with the exception of some of the in situ methods that utilize detergents. For example, hydrogenated Triton is a detergent that has been used for in-gel and on-membrane protein digestion. Although hydrogenated Triton is compatible with UV detection, its compatibility with MALDI-MS is more limited (Gharahdaghi et al., 1996). The highest-quality spectra are obtained from peptides that are purified by HPLC using volatile buffers. Peptide mixtures that are derived from a proteolytic digestion should be analyzed along with a protease control. The protease control should consist of both the same amount of enzyme and the same conditions that were used to digest the sample. Analysis of the enzyme control allows identification of protease autolytic fragments that can interfere with sample analysis. These fragments may be difficult to identify without the use of a protease control because some enzymes are modified to increase stability. Table 16.2.3 lists the commonly observed autolytic fragments for trypsin and lysine-C.

ANALYSIS OF SYNTHETIC PEPTIDES

Mass spectrometry has taken a leading role in the verification of proper synthesis of peptides. In particular, it is sensitive to identification of deletions, improper composition, unremoved protecting groups, scavenger adducts, oxidation, deamidation, and other undesired synthetic products. Amino acid analysis, although the traditional method for verification, cannot identify many of these problems. For this reason, mass spectrometry has replaced amino acid analysis as the routine method for synthetic peptide analysis in many synthesis labs. Crude samples can be analyzed successfully by MALDI. Samples are typically prepared to yield a peptide concentration of 1 to 50 pmol/µl. If the quantity is not known, two or three 10-fold dilutions of the crude peptide sample will usually yield a suitable concentration. Care should be taken in interpreting the results when extremely acid-labile protecting groups are used because these may be removed by the acidic environment of the MALDI matrix.

A number of studies of peptide synthesis have been carried out by the Association of Biomolecular Resource Facilities (ABRF); in these studies various ionization techniques

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were evaluated for their utility in peptide analysis (Fields et al., 1994, 1995). In comparisons with electrospray ionization (ESI), MALDI gave nearly identical results for identification of improperly synthesized or improperly deprotected peptides. Approximate determination of the relative amount of properly synthesized material was also nearly the same for MALDI and ESI and correlated well with HPLC analysis. MALDI should not normally be relied upon for quantitation unless appropriate internal standards are used. Nonetheless, in the case of synthetic peptides, the approximate relative amounts of product/byproducts can often be determined successfully because the byproducts are very similar in sequence and properties to the product.

When possible, the spectra should be obtained with sufficient resolution and mass accuracy to confirm that the correct peptides were prepared. In many cases, this requires resolving masses that differ by just 1 Da, with mass accuracy <0.5 Da, to eliminate the possibility of deamidation. When peptides are formed with disulfide bonds, the mass difference between the oxidized and reduced forms is only 2 Da. The mass measurement accuracy must distinguish between these two possibilities. Moreover, a mixture of reduced and oxidized forms often exists, and sufficient resolution is required to show the relative abundance of each form. On instruments in which the necessary accuracy or resolution is not possible to achieve, an alternative is to show reactivity of free sulfhydryls to alkylation, with the accompanying larger shifts in mass (Zaluzec et al., 1994; also see Analysis of Chemical Modifications).

ANALYSIS OF CHEMICAL MODIFICATIONS

MALDI-MS can provide rapid identification of peptide modifications. One modification that is commonly observed with this technique is oxidation of tryptophan and methionine residues. Oxidation of a tryptophan results in an increase of 32 Da and oxidation of methionine results in an increase of 16 Da (methionine sulfoxide); oxidation of methionine to methionine sulfone (+32 Da) is generally not observed. The presence of a +16- or +32-Da adduct can be used as an indirect indication of the presence of an oxidized methionine or tryptophan residue. Oxidation can occur during peptide purification, storage, or mass analysis. Thorough degassing of the HPLC mobile phases and use of nonmetal frits can minimize oxidation during purification. Storage at –5° or –80°C can minimize oxidation during sample storage. Because oxidation can also result when a peptide is stored with matrix on a sample stage, best results are obtained when the sample is analyzed immediately after the matrix has been added. Some peptides may be more susceptible to such oxidation than others.

A second type of modification that may occur during MALDI analysis is disulfide bond cleavage (Zhou et al., 1993; Patterson and Katta, 1994; Crimmins et al., 1995). The extent of disulfide cleavage may be greater at higher laser power. A protocol has been developed for detection of disulfide bonds using on-the-probe reduction (Fischer et al., 1993). After a spectrum is acquired, reducing agent is added to the sample stage, and the spectrum is acquired again. The peak for each disulfide-linked peptide then disappears, and new, smaller peaks appear whose masses when summed equal the mass of the oxidized peptide. The reducing agent should be carefully titrated because a large excess can cause severe ion suppression.

Another commonly observed modification is the formylation of amino groups (+28 Da) when formic acid is used as a solvent. Formic acid is a common solvent for cleavage at methionine residues using cyanogen bromide; however, other solvents—e.g., 0.1 N HCl or 70% trifluoroacetic acid—can be substituted. Amino-terminal glutamine is known to cyclize to pyroglutamic acid under certain aqueous conditions, and this reaction may also take place in the acidic environment of the matrix. Peptides containing cysteine that are derived from a protein separated by SDS-PAGE can be modified by free acrylic acid present in the gel. This adduct will add 71 Da to peptide mass. Proteins that are purified or refolded in the presence of urea may become carbamylated. Peptides can be carbamylated at the amino terminal and at lysine residues, resulting in a +43-Da increase for each carbamyl group added. Some of the more commonly observed chemical and post-translational modifications are listed in Table A.1A.3 (*APPENDIX 1A*). A more extensive listing of post-translational modifications and their corresponding mass shifts is available on the World Wide Web (see Table 16.2.4 for useful mass spectrometry sites). Future supplements will present detailed methods for MALDI-MS analysis of phosphorylation and glycosylation, two frequently observed post-translational modifications.

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Table 16.2.4 Useful Mass Spectrometry Web Sites

Site	Features	WWW URL
Delta Mass	Extensive list of mass shifts due to amino acid modifications	http://www.medstv.unimelb.edu.au/WWWDOCS/SVIMRDocs/MassSpec/deltamassV2.html
EMBL Protein Core Facility home page	Extensive information on database searching	http://www.mann.embl-heidelberg.de
Rockefeller/NYU mass spectrometry home page	Many features including a library of matrices	http://chait-sgi.rockefeller.edu
UCSF mass—spectrometry home page	On-line database searching	http://rafael.ucsf.edu
Murray's mass spectrometry home page	Best set of hyperlinks to other mass spectrometry web sites	http://tswww.cc.emory.edu/~kmurray/mslist.html
American Society for Mass Spectrometry home page	Information on ASMS meetings and short courses	http://www.trail.com/asms
Univ. of Washington (Ken Walsh's laboratory) home page	Programs for peptide digest interpretation	http://128.95.12.16/WalshLab.html
Univ. of Washington (John Yates' laboratory) home page	Information on database searching with fragmentation spectra	http://thompson.mbt.washington.edu
Pedro's BioMolecular Research Tools	A large collection of useful hyperlinks to mass spectrometry, protein chemistry, and molecular biology sites	http://www.public.iastate.edu/~pedro/research_tools.html

SUMMARY

Peptides as a class of compounds are extremely amenable to analysis by MALDI-MS. The high sensitivity, tolerance of many buffer components, rapidity of measurement, and ease of use of the technique have made it an indispensible tool for routine analysis of peptides from a wide variety of natural and synthetic sources. In particular, the direct measurement of molecular mass gives insight into modifications that are frequently not observed by most other techniques. Advances in instrumentation for time-of-flight analyzers are giving MALDI-TOF instruments the performance characteristics (resolution, mass accuracy, and fragmentation measurement) found previously only in other types of mass spectrometers.

The other major mass spectrometry technique for peptide analysis, electrospray ionization (see *UNIT 16.1*), has many characteristics similar to MALDI-MS. Electrospray offers the advantage of on-line coupling with chromatographic and electrophoretic techniques, but MALDI-MS displays a higher tolerance for buffer components, often results in higher sensitivity, and is operationally a bit easier to use. Nonetheless, both techniques are equally applicable to a large variety of problems and they complement each other in many ways. The choice of technique frequently depends on the particular problem at hand.

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STRUCTURAL BIOLOGY

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CHAPTER 17
Structural Biology

INTRODUCTION

Proteins have evolved to perform specific functions which depend on their three-dimensional structure. These three-dimensional structures are directly related to amino acid sequences (**primary** structure) of the proteins. Different segments of the primary structure form units of **secondary** structure, such as α helices or β pleated sheets. The **tertiary** structure refers to the three-dimensional arrangement of all the atoms that constitute a protein molecule. It is defined by the precise spatial coordination of the component secondary structural elements. The tertiary structure of proteins can usually be divided into **domains** which are comprised of distinct compact folding units usually containing about 100 to 200 amino acid residues. Small proteins may contain only a single domain, whereas most proteins contain multiple domains. A **fold** refers to the characteristic spatial assembly of secondary structure elements that comprise a domain; they are usually common to many proteins, e.g., the immunoglobulin fold. In *UNIT 17.1*, the authors give an overview of protein structural characteristics and provide an extensive survey of common structural folds that form the building blocks of the three-dimensional structures of proteins. For each fold described, the salient structural features are described, usually accompanied by illustration.

Several technologies are employed to study the three-dimensional structures of proteins. Electron microscopy (EM) can be utilized in protein structural analyses (*UNIT 17.2*). Although EM has long been employed to visualize the substructure of cells and tissues and the spatial array of proteins within them, it has not been extensively utilized for the study of proteins in general. The goal of this unit is to give a general overview of current EM procedures for observing proteins and to outline the kind of information that may be acquired by illustrating this with specific examples. The premise is that EM is complementary to—not in competition with—more established techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) for the study of three-dimensional structure.

UNIT 17.3 deals with the principles of macromolecular X-ray crystallography. The first part of the unit provides an overview of the underlying principles upon which this technology is based. It discusses the generation of the diffraction pattern, data collection, phase determination, resolution, refinement, and the Ramachandran plot, which is used by crystallographers to assess the quality of their structure determinations and to detect errors in the tracing of the polypeptide chain. Appreciation of these parameters should allow one to critically assess the quality of reported crystal structures. In the second part of the unit, the methods and concerns for preparing a protein sample for crystallographic studies are described. Included are details about preparation, assessment of quality and complexity, and storage of the protein samples. Procedures for setting up initial crystallization trials are described with suggestions for modifying these trials should they be unsuccessful. Finally, for cases where all else has failed to gain useful crystals, protein engineering strategies are discussed using HIV-1 integrase as a model protein for which these strategies have proven successful.

The major bottleneck in structural determination by X-ray crystallography is no longer a lack of sufficiently pure material—this problem has largely been eliminated by producing

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proteins with recombinant technology—but instead the preparation of suitable crystalline samples. Advances in procedures for macromolecular crystallization, combined with the availability of commercial screening kits, have made it possible to set up crystallization screening trials in a conventional biochemistry laboratory. This is particularly desirable given that crystallization of the protein will generally enhance an investigator's chances of attracting a crystallographic collaborator. *UNIT 17.4* outlines the steps for accomplishing this starting with a purified sample. Protocols are given for concentrating the sample, for preparing and assessing the screening trials, and finally for optimizing the crystallization conditions.

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Overview of Protein Structural and Functional Folds

ELEMENTS OF PROTEIN STRUCTURE

All proteins are unbranched polymers of L-amino acids that fold into stable three-dimensional shapes or conformations unique to a particular protein. The complete structure of a protein can be described at four different levels of complexity: primary structure, secondary structure, tertiary structure, and quaternary structure.

Primary Structure

The primary structure of a protein is defined as the linear amino acid sequence of a protein's polypeptide chain. The term **protein sequence** is often used interchangeably with primary structure. As demonstrated by Chris Anfinsen (Anfinsen, 1973), the primary amino sequence of a protein uniquely determines the higher orders of structure for a protein and is thus of fundamental importance.

Secondary Structure

Secondary structure can be defined as the local spatial conformation of the polypeptide's backbone, excluding the side chains. Regular secondary structures (also referred to as **secondary structure elements**) common to many proteins include α helices, β sheets, and turns. These can vary widely in length, from as few as three to five residues in short helices and sheets to over fifty residues in some coiled-coil helices. Such structures are generally defined both by characteristic main-chain φ and ψ dihedral angles (the torsion angles between backbone atoms C_{i-1}–N_i–C_i^{α}–C_i and N_i–C_i^{α}–C_i–N_{i+1}, respectively; see Fig. 17.1.1A) and by regular main-chain hydrogen-bonding patterns. The term **topology** is often used to refer to the connectivity of secondary structure elements. For example, a portion of a protein containing a β strand connected to an α helix and then another β strand is said to have a βαβ topology. The complete secondary structure of a polypeptide chain is often represented in two dimensions by a topology diagram that shows both the connectivity and the relative orientation of neighboring secondary structure elements. Such diagrams are particularly useful in classifying β sheets.

An α helix is formed when a protein backbone adopts a right-handed helical conformation with 3.6 residues per turn and a set of hydrogen bonds formed between the main-chain CO of the i^{th} residue and the main-chain NH of the $(i + 4)^{th}$ residue (Fig. 17.1.1B). Occasionally, hydrogen bonds are observed between residues i and $i + 3$, resulting in a 3_{10} helix. Compared to the more common α helices, 3_{10} helices are much shorter, usually comprising only one to three turns. The optimal backbone φ and ψ dihedral angles are, approximately, (–57, –47) degrees for a right-handed α helix, and (–49, –26) degrees for a 3_{10} helix.

β sheets (also referred to as β pleated sheets) make up another major secondary structure element in proteins. A β sheet consists of at least two β strands with each one approaching an extended backbone conformation and dihedral angles confined to a region of $-180° < \phi < -60°$, $30° < \psi < 180°$. All main-chain CO-to-NH hydrogen bondings are between adjacent strands (Fig. 17.1.1C). A parallel β sheet is formed when all the sheet-forming strands are running parallel to each other from N to C termini, whereas an antiparallel β sheet is formed when the strands are antiparallel to each other (Fig. 17.1.1C). A β sheet formed from both parallel and antiparallel strands is referred to as a mixed β sheet. A characteristic feature of β sheets is the right-handed twist, which is visible when the sheet is viewed edge-on (Fig. 17.1.1D).

Loops and **turns** connect α helices and β sheets in protein structures. Except for two common types of tight turns connecting adjacent antiparallel β strands, most turns and loops assume irregular secondary structures. The type I and II **reverse turns** (also referred to as β turns or β bends; Fig. 17.1.1E) each have a hydrogen bond between the CO of the first residue in a turn and the NH of the fourth residue (also the last residue) in the turn. The difference between the type I and type II turn is a 180° flip of the peptide plane in the middle of the turn. The type I turn is more frequently observed than the type II turn. Loops and coils generally refer to secondary structure elements that display less regular hydrogen bonding patterns than those observed in α helices, β sheets, and reverse turns.

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Tertiary Structure

Tertiary structure refers to the three-dimensional arrangement of all the atoms that constitute a protein molecule. It relates the precise spatial coordination of secondary structure elements and the location of all functional groups of a single polypeptide chain.

The tertiary structure of a protein can often be divided into **domains,** which are distinct and compact folding units usually comprising 100 to 200 residues. Small proteins may contain a single domain, whereas larger proteins often contain multiple domains. A **fold** refers to a characteristic spatial assembly of secondary structure elements into a domain-like structure that is common to many different proteins. Particular folds are often, though not always, related to a certain function (e.g., nucleotide-binding folds). A **structural motif** is similar to a fold, but is generally smaller, tending to form the "building blocks" of folds. Some structural motifs (e.g., β barrels) are observed with many variations in a vast array of unrelated proteins, whereas others, especially those related to a unique biochemical function (e.g., zinc fingers), are highly conserved, generally occurring in protein domains of similar function. Highly conserved structural motifs often display a characteristic signature at their amino acid sequence level—the so-called **sequence motif.** Very often the term **motif** is used by itself to refer to either a **structural motif** or a **sequence motif.** This can lead to confusion because structural motifs do not always have a unique amino acid signature. The terms domain, fold, and motif are often used interchangeably with blurred distinctions. This happens especially for larger, highly conserved structural motifs that may be equally well known as a particular type of domain or fold.

Quaternary Structure

Many proteins, especially those >100 kDa in mass, are oligomers consisting of more than one polypeptide chain. The precise spatial arrangement of the subunits within a protein is referred to as the quaternary structure.

Frequently Observed Secondary Structure Assemblies/Structural Motifs

Below are described some structural motifs commonly observed in a wide variety of proteins. Other structural motifs of particular functional significance, such as DNA- and RNA-binding motifs, are described in later sections.

The term **helix bundles** (or **helical bundles**) usually refers to the packing of several helices in a nearly parallel or antiparallel orientation. In some cases, the term helix bundles also applies to the general packing of helices regardless of their orientation to each other. There are many examples of protein structures that contain three, four, or multiple helix bundles—e.g., cyclin (3-helix bundle), hematopoietic cytokines (4-helix bundle), globins (6-helix bundle), and cytochrome *c* oxidase (22-helix bundle). Helix bundles (Fig. 17.1.2A) are often distinguished by the number of helices in the bundle, the relative direction of the helices, and the characteristic twist of each helix bundle. In describing the direction of the helices in a bundle, both "parallel-antiparallel" and "up-down" terminologies are used in the literature, although the latter convention gives a more precise description. In the former convention, two helices are said to be parallel if their N- to C- termini vectors or peptide directions are parallel to each other; the opposite orientation is referred to as antiparallel. In the "up-down" notation, the first helix in a bundle is defined as the "up" helix, and all subsequent helices are assigned either "up" or "down" depending on whether they are parallel or antiparallel to the first helix. In most helical bundles, the axes of individual helices are inclined to the representative axis of the entire bundle in a systematic manner, forming a "twist" with defined handedness that resembles a spiral. Most helix bundles carry a left-handed twist.

As a special case of helix bundles, **coiled coil** refers to two or more helices intertwined with each other to form a supercoiled helical structure. The helices involved in coiled-coil configurations are normally long helices with certain characteristic repeats in their amino acid sequences, and they are usually parallel rather than antiparallel to each other. In many cases, the participating helices of a coiled coil are from separate subunits but reside in a topologically equivalent part of a protein.

When an antiparallel β sheet contains only two strands connected by a single tight turn, it is termed as a β hairpin structure (Fig. 17.1.2B). A **β hairpin** (or β ribbon) is usually highly twisted and less regular compared to a more extended β sheet.

In an antiparallel β sheet assembly, four adjacent strands are sometimes arranged in a pattern similar to the repeating unit of one of the ornamental patterns used in ancient Greece (Fig. 17.1.2C). Thus this type of strand assembly is termed a **Greek key** motif. The immunoglobulin fold is one example of a fold that contains the Greek key motif. A certain arrange-

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ment of two Greek key motifs form the so-called **jelly roll** (Fig. 17.1.2D). In this case, strands 1, 2, 7, and 8 form one Greek key; strands 3, 4, 5, and 6 form the other. The eight strands fold into a nearly closed barrel shape. A characteristic feature in the topology of any β sheet is the arrangement of all the strands in the sheet—i.e., the strand order within the hydrogen bonding of the sheet. This hydrogen bonding–directed strand order is also called strand connectivity. In the case of a jelly roll motif, this strand connectivity has an order of 1–2–7–4–5–6–3–8.

The term **β sandwich** refers to a set of β strands arranged into two β sheets packed face-to-face against each other (Fig. 17.1.2E). The two sheets can be made out of parallel, antiparallel, or mixed strands. In many β sandwiches, the strands of the two sheets are oriented either parallel/antiparallel or approximately orthogonal with respect to each other. A common feature in some types of β sandwiches is **strand switching**, which involves an edge strand that hydrogen bonds to one sheet at its N-terminal end and to the other sheet at its C-terminal end.

Aside from the β sandwich, other layered secondary structure assemblies include α/β, α/β/α, and β/β/α **sandwiches**. For example, an α/β/α sandwich is defined as a structure with one layer of β sheet (middle layer) sandwiched in between two layers of α helices. Both layers of helices pack closely against the middle β sheet layer. The α/β/α sandwich is commonly observed in nucleotide-binding proteins.

β barrels are assemblies of β strands folded into closed barrel-like structures as viewed from the ends of the strands (Fig. 17.1.2F). The size and shape of a barrel varies widely in protein structures. A given barrel can have as few as five strands—as in the src homology SH3 domain—or as many as sixteen strands—as observed in the structure of porin. The strands can be arranged in parallel, antiparallel, or mixed orientations.

In contrast to a β barrel, which consists of entirely β strands, an **α/β barrel** is made out of alternating α and β structures. A typical example of an α/β barrel fold is shown in the structure of the triosephosphate isomerase (TIM), which has eight repeats of an alternating unit and consists of a β strand followed by an α helix (Fig. 17.1.2G). It is organized such that the eight β strands form an inner β barrel, which is then surrounded by eight α helices. α/β barrels are frequently observed in enzyme structures. See discussion of Enzymes, below, for a more detailed description of TIM barrel structure.

ANALYSIS OF PROTEIN FOLDS

As a multitude of protein structures are being determined by X-ray crystallography and nuclear magnetic resonance (NMR) at exponential rates, it is becoming clear that the number of unique folds is far less than the total number of protein structures. Not only do functionally related proteins generally have similar tertiary structures, but even proteins with very different functions are often found to share the same tertiary folds. As a consequence, structural conservation at the tertiary level is perhaps more profound than it is at the amino acid–sequence level. The identification of the fold of a protein has therefore become an invaluable tool because it can potentially provide a direct extrapolation to the function and may allow one to map out functionally important regions in the amino acid sequence.

Several groups have already attempted to classify protein structures into families and superfamilies of folds without focusing on function (Orengo and Thornton, 1993; Orengo et al., 1993, 1994; Murzin et al., 1995). The scope of this review is not to enumerate all the existing folds and tertiary structures determined to date, but rather to provide a comprehensive overview of some commonly observed protein-fold families and commonly observed structural motifs that have functional significance. The entire overview is organized into sections based on both structural and functional relations. For purposes of clarity, the proteins mentioned in the text or in figures are arranged in tables with their references under appropriate structural headings.

INTERNET ACCESS TO PROTEIN DATA BANK FILES

The Brookhaven Protein Data Bank (PDB; Bernstein et al., 1977; also see Chapter 2) maintains a structural archive for any atomic-resolution protein structure determined primarily by X-ray crystallography and nuclear magnetic resonance techniques. The structures are stored as sets of three dimensional (x,y,z) positional coordinates for all the atoms of each structure. These coordinate files are written in PDB format, which can be read and displayed by a number of existing graphics programs. The simplest method for retrieving a structural coordinate file is to obtain it from the PDB World Wide Web site *http://www.pdb.bnl.gov/* using Internet access software such as Netscape or Microsoft Internet Explorer. As an example, the following steps are outlined for retrieving the structure of a hemoglobin from the PDB homepage.

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1. Connect to the PDB homepage at *http://www.pdb.bnl.gov/* and choose "Searching and Browsing the PDB."

2. Choose "3DB Browser" within the Searching and Browsing the PDB page to open a browser menu.

3. Input the word "hemoglobin" in the full text query window and choose "Start a Search." This search will result in a list of entries matching the query "hemoglobin" with one entry per line. Highlight one and select "Retrieve Data" to go to the data-retrieval menu.

4. Choose "Complete with Coordinates" to start coordinate retrieval. Once the coordinates of a given structure are retrieved, they can be displayed by any graphics programs that are capable of reading PDB files. Programs such as RASMOL, KINEMAGE, QUANTA, O, MOLSCRIPT, SETOR, and RIBBONS can all be used to view structures. Both RASMOL and KINEMAGE can be downloaded without cost from the PDB WWW site including versions for PC, Macintosh, DEC, and SGI computers. All ball-and-stick models and ribbon diagrams shown in Figures 17.1.2 to 17.1.37 are created from PDB coordinates using the program MOLSCRIPT (Kraulis, 1991).

Two other Web-based programs—SCOP (*http://www.pdb.bnl.gov/scop;* Murzin et al., 1995) and CATH (*http://pdb.pdb.bnl.gov/bsm/cath*), allow access to the PDB through a hierarchical classification of protein structures.

SURVEY OF STRUCTURAL AND FUNCTIONAL FOLDS

Immunoglobulins and the Immunoglobulin-Like Fold

Traditionally, the term immunoglobulin (Ig) fold refers to the homologous domain structures present in the variable and constant regions of all immunoglobulins. In recent years, however, with the rapid growth of sequence and structure knowledge, many other proteins whose functions are not related to immunoglobulins have been found to contain one or more Ig-like domains in their tertiary structures. Examples of such proteins include the cell-surface glycoprotein receptors such as growth hormone receptor, CD4 and CD8, adhesion molecules such as cadherins and type III fibronectins, class I and class II major histocompatibility proteins, chaperone PapD, the transcriptional factor NF-κB and even some enzymes such as Cu and Zn superoxide dismutase and β-galactosidase (Bork et al., 1994; Murzin et al., 1995). The discovery of the Ig-like domains in many proteins not only broadens the definition of the superfamily, but more importantly demonstrates the versatility of the fold and the ability of these domains to function as modular units in many diverse systems.

The immunoglobulin fold is defined as a domain that is formed from seven to nine β strands folded into a Greek key β sandwich (Fig. 17.1.2A). The topology consists of one β sheet formed by strands A, B, E, and D; the opposing sheet is formed by strands C, C′, (occasionally) C″, F, and G. Based on the strand hydrogen bonding pattern and the number of strands in each domain, the superfamily is further divided into five commonly observed subclasses: V, C1, C2, I, and E (Fig. 17.1.3; Williams and Barclay, 1988; Murzin et al., 1995). The V-type domain (Fig. 17.1.3A) has the same topology as immunoglobulin variable (V) regions. It has nine strands with a typical "strand switch" occurring in the first strand. The strand switch usually breaks into two halves (A and A′) with the first half (strand A) hydrogen bonding to strand B of the B–E–D β sheet and the second half (strand A′) hydrogen bonding to strand G of the opposing β sheet. The additional strands C′ and C″ are usually shorter than the other strands. In immunoglobulins, the antigen-binding site is composed primarily of three loop regions termed complementary determining regions (CDRs). They are located between strands B and C, strands C′ and C″, and strands F and G. The C1 type of immunoglobulin fold (Fig. 17.1.3B) refers to the topology observed in the structure of constant regions of immunoglobulins. It has seven strands, of which strands A–B–E–D form one β sheet and strands C–F–G form the other. Most members of the C2-type subclass (Fig. 17.1.3C) have seven strands and differ from the C1 type by a simple switch in strand D from hydrogen bonding with strand E in the A–B–E sheet to hydrogen bonding with strand C in the C–F–G sheet. Hence strand D is labeled as strand C′ in the C2 type subclass. Some members of the C2 family also have additional strands at either the N or C termini. Based on tertiary-structure similarities, there are two frequently adopted tertiary folds in the C2 class; one is similar to the C2 domain of T lymphocyte cell surface glycoproteins CD2 and CD4 and the other is similar to the fibronectin type III domain. The latter fold is also referred to as the fibronectin type III–like fold, shown as C2(Fn)

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in Figure 17.1.3C. The I-type domains (Fig. 17.1.3D), recently proposed by Harpaz and Chothia (1994), closely resemble the topology of immunoglobulin variable domains, except that they lack strands C′ and C″. The members of the I-type subclass also have the characteristic "strand switching" phenomena in the first strand, as do the immunoglobulin variable domains. The E-type immunoglobulin domains (Fig. 17.1.3E) also present a topology similar to those of the V-type except that they lack the C″ and D strands. Table 17.1.1 summarizes all the observed immunoglobulin subclasses and lists references for specific proteins.

The most recent structure determination of an αβ 2C T cell receptor (TCR) at 2.5-Å resolution has revealed an unusual variant of the immunoglobulin C1 type—the constant Cα domain of the receptor. While the typical C1 type has strands A, B, E, and D forming one β sheet and strands C, F, and G forming the opposing sheet, the first β sheet in the Cα domain of 2C TCR is formed by strands G, A, B, E, and D, with strand G hydrogen bonding to strand A. The opposing β sheet, which is normally formed by strands C, F, and G, displays a quite different conformation as observed in C1 type. Particularly, strand F has a one-turn α helix in the middle and strand C becomes largely a loop-like structure.

Among members of the immunoglobulin superfamily, occasionally, additional strands are observed at the N and C termini, and insertion strands that lie outside the core immunoglobulin fold can sometimes be found connecting the adjacent immunoglobulin strands.

MHC Peptide-Binding Fold

The three-dimensional structures of the class I and class II MHC molecules revealed a distinct fold associated with the peptide-binding domain of the major histocompatibility complex (MHC) molecules (see Table 17.1.2 for specific references). More recently, the crystal structure of a neonatal Fc receptor has provided another example to the family of the MHC fold. Although the two membrane-proximal domains of MHC molecules (the α3 and β2m domains of class I and the α2 and β2 domains of class II molecules) display an immunoglobulin fold (see Immunoglobulins and the Immunoglobulin-Like Fold), this section summarizes the structure of the MHC peptide-binding groove. In class I molecules, the peptide-binding domain is formed by the α1 and α2 regions of the heavy chain, whereas in class II molecules it is formed by the α1 and β1 regions of both the α and β chains (Bjorkman and Burmeister, 1994; Stern and Wiley, 1994; Madden, 1995). The peptide-binding domain consists of two α helices (one from each region) forming the two sides of the groove with an eight-stranded antiparallel β sheet (four strands from each region) forming the floor of the groove (Fig. 17.1.4). The size of the groove is ~30-Å long and 12-Å wide in the middle. Both class I and class II MHC molecules bind peptides in extended conformations with anchor residues buried inside pockets of the groove. The distribution of these pockets along the binding site determines the peptide selectivity of the particular allele of class I or class II molecules (Matsumura et al., 1992; Engelhard, 1994; Parker et al., 1995). An important structural difference between the class I and class II molecules that contributes to the peptide-length restriction is found at the ends of the binding groove. In class I MHC molecules, the ends of the peptide-binding groove are blocked with bulky aromatic residues and with specific hydrogen bonding networks that interact with the N and C termini of the peptides, thereby rendering class I molecules suitable for binding shorter peptides of an optimal length of eight or nine residues. However, longer peptides do bind to class I molecules, either by bulging out in the middle or by protruding through the ends. In contrast, both ends of the class II peptide-binding groove are open, allowing longer peptides to extend out of the binding groove; consequently, there is no physical constraint on the length of peptide that can bind.

Proteins Involved in Signal-Transduction Pathways

Table 17.1.3 contains references for specific proteins.

Cytokines

Four-helix bundles. Hematopoietic cytokines possess a common four-helix bundle topology in their structures. The four helices (labeled as a, b, c, and d) always pack in a left-handed up-up-down-down manner—with helices a and b and c and d, respectively, forming two pairs of parallel helices juxtaposed in opposite directions (Sprang and Bazan, 1993; Wlodawer et al., 1993). The positions of the four helices and the packing angles between them are generally well conserved. The superfamily is further divided into long-chain, short-chain, and interferon (IFN)-γ-like families of cytokines.

Members of the **long-chain family** of cyto-

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Table 17.1.1 Immunoglobulin Superfamily of Proteins

Protein	Immunoglobulin subtype	PDB entry	References
Immunoglobulins, antibodies		3hfl	
Variable region	V-type		
Constant region	C1-type		
Major histocompatibility complexes (MHC)		2clr	See Table 17.1.2
Class I heavy chain α3 domain	C1-type		
Class I β2 microglobulin	C1-type		
Class II α chain, C-terminal domain	C1-type		
Class II β chain, C-terminal domain	C1-type		
Cell-surface immunoglobulin-like receptors			
T cell receptor, αβ type			
Vα, Vβ domains	V-type		Garcia et al., 1996. *Science* 274:209-219
Cβ domain	C1-type		Bentley et al., 1995. *Science* 267:1984-1987
CD8 T cell coreceptor	V-type	1cd8	Leahy et al., 1992. *Cell* 68:1145-1162
Neonatal Fcγ receptor		1fru	Burmeister et al., 1994. *Nature* 372:336-343
Heavy chain α3 domain	C1-type		
β chain	C1-type		
Growth hormone receptor domain 1 and 2	C2(Fn)	3hhr	deVos et al., 1992. *Science* 255:305-312
Prolactin receptor domain 1 and 2	C2(Fn)	S042	Somers et al., 1994. *Nature* 372:478-481
Interferon γ receptor	C2(Fn)	S043	Walter et al., 1995. *Nature* 376:230-235
CD4 T cell surface glycoprotein		3cd4	Ryu et al., 1990. *Nature* 348:419-426; Wang et al., 1990. *Nature* 348:411-418; Brady et al., 1993., *Science* 260:979-983
Domain 1 (strand A pairs with G only), 3	V-type		
Domain 2, 4	C2-like		
Coagulation factors			
Tissue coagulation factor, ectodomain	C2(Fn)	2hft	Müller et al., 1994. *Biochemistry* 33:10864-10870
Cell matrix and adhesion proteins			
Fibronectin type III domain	C2(Fn)	1fna	Main et al., 1992. *Cell* 71:671-678
Tenascin, repeats	C2(Fn)	1ten	Leahy et al., 1992. *Science* 258:987-991
Neuroglian, two Fn3 repeats	C2(Fn)		Huber et al., 1994. *Neuron* 12:717-731
CD2		1hnf	Jones et al., 1992. *Nature* 360:232-236
Adhesion domain	V-type		
C-terminal domain	C2-like		
VCAM-1		1vca	Jones et al., 1995. *Nature* 373:539-544
Domain 1	I-type		
Domain 2	C2-like		
Cadherin (strand A pairs with strand G)	C1-type	1nci	Shapiro et al., 1993. *Nature* 374:327-336
Muscle proteins			
Telokin	I-type	1tlk	Holden et al., 1992. *JMB* 227:840-851
Titin	I-type	1tnm	Pfuhl and Pastore, 1995. *Structure* 3:391-401

continued

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Table 17.1.1 Immunoglobulin Superfamily of Proteins, continued

Protein	Immunoglobulin subtype	PDB entry	References
Transcription factors			
NF-κB, p50 subunit		1nfk	Ghosh et al., 1995. *Nature* 373:303-310
First Rel homology domain (with insertion strands between C and C′, E and F)	E-type	1svc	Müller et al., 1995. *Nature* 373:311-317
Second Rel homology domain	E-type		
Enzymes with immunoglobulin-like domains			
Chaperone protein			
Pap D		3dpa	Holmgren and Brändén, 1989. *Nature* 342:248-251
Domain 1 (strand A pairs with B and G)	C2		
Domain 2 (one additional strand at C terminus)	C2		
Oxidoreductase			
Cu, Zn superoxide dismutase (one additional strand at N-terminus)	C2	2sod	Richardson et al., 1976. *JMB* 102:221-235
Galactose oxidase, C-terminal domain	E-type	1gof	Ito et al., 1991. *Nature* 350:87-90
Cel D cellulase (hydrolase), N-terminal domain	E-type	1clc	Juy et al., 1992. *Nature* 357:89-91
Cyclodextrin glycosyltransferase, domain E	E-type	1cdg	Lawson et al., 1994. *JMB* 236:590-600
Chitinase A (lyase), N-terminal domain	E-type	1ctn	Perrakis et al., 1994. *Structure* 2:1169
Other			
Hemocyanin (oxygen transporter)	E-type	1oxy	Magnus et al., 1994. *Proteins* 19:302-309
β-galactosidase, domains 2 and 4		1bgl	Jacobson et al., 1994. *Nature* 369:761-766
Synaptotagamin I			

Table 17.1.2 Major Histocompatibility Complex Proteins

Protein	PBD entry	Reference
Peptide-binding domains		
HLA-A2	2clr	Bjorkman et al., 1987. *Nature* 329:506-512
HLA-Aw6B		Garrett et al., 1989. *Nature* 342:692-696
HLA-B27		Madden et al., 1992. *Cell* 70:135-148
H-2K^b		Fremont et al., 1992. *Science* 257:919-927; Zhang et al., 1992. *PNAS* 89:8403-8407
HLA-DR1		Brown et al., 1993. *Nature* 364:33-39; Stern et al., 1994. *Nature* 368:215-221
Neonatal Fc receptor	1fru	Burmeister et al., 1994. *Nature* 372:336-343

kines include growth hormones (GH), granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), leukemia-inhibiting factor (LIF), IL-6, IL-11, IL-12, interferon β, and ciliary neurotrophic factor. The four helices of this family are each ~25 residues long (Fig. 17.1.5A and D).

The **short-chain family** includes most of the interleukins (IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, and IL-13), granulocyte/macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), and stem cell factor (SCF). In comparison to members of the long-chain family, the helices are shorter and uneven in length (8 to 15 amino acids long). Another signature of short-chain hematopoietins is the presence of a pair of short, twisted antiparallel β strands, one in the loop

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Table 17.1.3 Signal-Transducing Proteins

Protein and motif	PDB entry	References
Cytokines		
Four-helix bundles		
Long chain, G-CSF	1rhg	Hill et al., 1993. *PNAS* 90:5167-5171
Short chain		Rozwarski et al., 1994. *Structure* 2:159-173
Interleukin 4	1rcb	Smith et al., 1994. *Nature Struct. Biol.* 1:301-310
Interferon γ–like		
Interferon γ	1rfb	Ealick et al., 1991. *Science* 252:698-702
Interleukin 10		Zdanov et al., 1995. *Structure* 3:591-601
β trefoil		
Interleukin 1α		Hendrickson et al., 1990. *Biochem.* 29:2679-2684
Interleukin 1β	1hib	Priestle et al., 1989. *PNAS* 86:9667-9671; Clore and Gronenborn, 1991. *JMB* 221:47-53
Fibroblast growth factor		Eriksson et al., 1991. *PNAS* 88:3441-3445; Zhang et al., 1991. *PNAS* 88:3446-3450; Zhu et al., 1991. *Science* 251:90-93
Cysteine-knot growth factors		
Transforming growth factor β	2tgi	Daopin et al., 1992. *Science* 258:1160-1161; Daopin et al., 1992. *Proteins* 17:176-192; Swindells, 1992. *Science* 258:1161; Murray-Rust et al., 1993. *Structure* 1:153-159; Schlunegger and Grütter, 1993. *JMB* 231:445-458
Nerve growth factor	1bet	McDonald et al., 1991. *Nature* 354:411-414; Daopin et al., 1992. *Science* 258:1160-1161; Swindells, 1992. *Science* 258:1161; Murray-Rust et al., 1993. *Structure* 1:153-159
Platelet-derived growth factor BB	1pdg	Oefner et al, 1992. *EMBO J.* 11:3921-3926; Murray-Rust et al., 1993. *Structure* 1:153-159
Human chorionic growth factor	1hcn	Lapthorn et al., 1994. *Nature* 369:455-461; Wu et al., 1994. *Structure* 2:545-558
Epidermal growth factor–like		
Epidermal growth factor	1epj	Kohda and Inagaki, 1992. *Biochem.* 31:11928-11939; Rao et al., 1995. *Cell* 82:131-141
E-selectin		Graves, et al., 1994. *Nature* 367:532-538
Receptors		
Aspartate	1wat	Milburn et al., 1991. *Science* 254:1342-1347
Integrin A domain	1lfa	Qu and Leahy, 1995. *PNAS* 92:10277-10281; Lee et al., 1995. *Cell* 80:631-638
Low-density lipoprotein	1ldl	Daly et al., 1995. *PNAS* 92:6334-6338
Tumor necrosis factor	1ncf	Banner et al., 1993. *Cell* 73:431-445
Modular domains		
SH2 domain	1sha	Waksman et al., 1992. *Nature* 358:646-653; Kuriyan and Cowburn, 1993. *Curr. Opin. Struct. Biol.* 3:828-837; Mikol et al., 1995. *JMB* 254:86-95
SH3 domain	1csk	Musacchio et al., 1992. *Nature* 359:851-855; Yu et al., 1992. *Science* 258:1665-1668
PH domain	1dyn	Gibson et al., 1994. *TIBS* 19:349-353; Macais et al., 1994. *Nature* 369:675-677; Yoon et al., 1994. *Nature* 369:672-675; Ferguson et al, 1995. *Cell* 83:1037-1046

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connecting helices a and b and the other in the loop connecting helices c and d (Fig. 17.1.5B and D). Many of them also have disulfide bridges in their structures. However, the location of these disulfide bonds are not well conserved.

Interferon γ–like. This family includes IFN-γ and IL-10. Both of these cytokines contain six helices (a, b, c, d, e, and f) and function as homodimers. The dimerization is particularly interesting in this family. The two domains of the dimer are formed by swapping the last two helices (e and f) between the two monomers. The four-helix bundle–forming helices in each domain are made of helices a, c, and d from one monomer and helix f from the other monomer (Fig. 17.1.5C and E).

β trefoil. Members of the **β trefoil** family include not only cytokines such as interleukin 1α, interleukin 1β, and fibroblast growth factor, but also proteins such as soybean trypsin inhibitor and hisactophilin which display the same fold. The overall structure forms a 12-stranded closed β barrel with an internal pseudo-three-fold symmetry (Fig. 17.1.6A) relating three four-stranded antiparallel β sheets. Each β sheet is made of four consecutive strands. For example, the three β sheets of IL-1β are composed of strands L–A–B–C, D–E–F–G, and H–I–J–K, respectively.

Cysteine-knot superfamily. The recent crystal structures of four different growth factors—nerve growth factor (NGF), transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), and human chorionic gonadotropin (hCG)—have revealed a common overall topology among these functionally diverse families of proteins (Murzin and Chothia, 1992; Sun and Davies, 1995). These proteins share very little sequence homology. Nevertheless, they all have an unusual arrangement of six cysteines that are disulfide-linked to form a knotted conformation, and this core cysteine-knot is flanked by four strands of an antiparallel β sheet. Figure 17.1.6B shows the crystal structure of human TGF-β2 with the common flanking β strands (1, 2, 3, and 4) labeled and the six disulfide-linked cysteines shown in ball-and-stick models. Outside the four structurally conserved β strands, the helices and loops observed in the structure of TGF-β2 are not conserved in NGF, PDGF, and hCG. The three disulfide bonds formed between the six cysteines of TGF-β2 are Cys15–Cys78, Cys44–Cys109, and Cys48–Cys111—a linkage pattern of I–IV, II–V, and III–VI among the six cysteines. A topological knot is formed by threading the disulfide I–IV through a ring formed with disulfide II–V, III–VI as its two sides and the peptide in between cysteine II and III, V and VI as its other two sides. Another common structural feature among the cysteine-knot cytokines is that the active forms of these proteins are always dimers, either homo- or heterodimers. However, despite the overall topological similarity between the monomers, each family of growth factors forms its own distinct dimers with a unique, characteristic dimer interface.

EGF-like domain. EGF-like domains are often observed in tandem repeats in many proteins involved with a receptor-related signal-transduction component (Groenen et al., 1994). Members of this family include epidermal growth factor (EGF), transforming growth factor α (TGF-α), EGF domain of E-selectin, Factor X, Factor IX, plasminogen activator, prostaglandin H2 synthase 1, and thrombomodulin. An EGF module has ~50 amino acids and is stabilized by three disulfide bonds. Its structure elements consist of two small β sheets—a three-stranded antiparallel β sheet close to the N terminus and a two-stranded antiparallel β sheet near the C terminus (Fig. 17.1.6C). The disulfide bonds are formed between cysteines I–III, II–IV, and V–VI in sequence.

Receptors

Elucidating the atomic structures of cell-surface receptors is a key step toward understanding receptor-mediated signal transduction. A number of receptor structures have been determined in recent years. Table 17.1.4 lists the receptors whose structures are known to date. This section will review the non–immunoglobulin type receptors (also see discussion of Immunoglobulins and the Immunoglobulin-Like Fold).

Aspartate receptor–ligand binding domain. Aspartate receptor folds into a four–α helix bundle with the N- and C-terminal helices longer than the other two helices. The four-helical bundle has an up-down-up-down topology and a left-handed twist (Fig. 17.1.7A). The receptor functions as a disulfide-linked dimer with its ligand aspartate binding to a pocket in the receptor-dimer interface.

Tumor necrosis factor receptor–ligand binding domain. The structure of a human 55-kDa tumor necrosis factor (TNF) receptor shows a unique fold consisting of four homologous cysteine-repeat domains. There are no helices and very few regular β sheets in its

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Table 17.1.4 Receptor Families

Family	Fold of the ligand-binding domain
Immunoglobulin receptors—FcγR, FcεR, BCR	Immunoglobulin
T cell antigen receptor (TCR)	Immunoglobulin
Adhesion molecules	
Integrin-I domain	Flavodoxin-like
Cadherins	Immunoglobulin
Cytokine receptors	
Growth hormone receptor	Immunoglobulin
Tumor necrosis factor receptor	Cysteine-rich, β-hairpin repeats
Nuclear receptors	Nuclear receptor
Aspartate receptor	Four-helical bundle
LDL receptor	Cysteine-rich repeats

secondary structure (Fig. 17.1.7B). Instead, the fold is composed mostly of loops and β hairpins. Upon complex formation with TNF, there are three receptor molecules symmetrically bound to a TNF trimer.

Low-density lipoprotein receptor. Low-density lipoprotein (LDL) receptor belongs to a family of cysteine-rich repeats that bind to lipoproteins. The structures of the first and second repeat have been solved by NMR spectroscopy (Fig. 17.1.7D). Each repeat has mostly irregular secondary structure with one β hairpin.

Integrin A and I domain. The recently reported structures of the I domain from CD11b and CD11a integrin α chain have showed that these domains possess a flavodoxin-like fold similar to that seen in members of the dinucleotide-binding family of proteins. The tertiary structure consists of six-stranded, mostly parallel β sheets with only one antiparallel strand on one edge of the sheet (Fig. 17.1.7C). The connectivity of the strands follows a 3–2–1–4–5–6 order. Flanking the central β sheet are several α helices on each side. A bound divalent metal ion at the end of the β sheet is postulated to play an important role in its activity.

Modular domains involved in signal transduction

Many components of cellular signaling pathways, such as protein tyrosine kinases (PTKs), phosphatases (PTPases), subunits of phospholipases, adaptor protein Grb2, and cytoskeletal proteins contain small modular domains with conserved sequences (Pawson and Schlessinger, 1993; Pawson, 1995). Of these compact modules, three of them—Src homology 2 (SH2), Src homology 3 (SH3), and pleckstrin homology (PH) domains—are reviewed here. All three function as nonenzymatic adaptors to initiate complex protein-protein interactions. Proteins containing these domains generally have one or more copies of either individual type or a mix of different types of modules in their sequences. These domains are reviewed together, not because of their tertiary folds, but rather because of their relationship in function and their modular design nature.

SH2 domains. SH2 domains are primarily involved in cytoplasmic tyrosine kinase signaling pathways. Proteins with SH2 domains include protein tyrosine kinases (PTKs), phosphatases (PTPases), subunit of phospholipase C (PLC-γ1), p85 subunit of phosphatidylinositol-3OH kinase, cytoplasmic tail of cell-surface receptor proteins, and adaptor proteins like Grb2. The function of this module is to bind phosphotyrosine peptides.

The fold of an SH2 domain is characterized as an α/β barrel-like sandwich with a central core formed by a four-stranded antiparallel β sheet followed by a smaller three-stranded antiparallel β sheet (Fig. 17.1.8A). The β barrel is flanked by two α helices, one on each side of the larger β sheet. A phosphopeptide-binding site is located on a surface formed by the fourth strand of the central β sheet, the first helix, the loop between strands 5 and 6, and the loop at the end of helix 2. The phosphate moiety of the phosphotyrosine forms an ion pair with an arginine residue from the second strand of the central β sheet. Another arginine from the first helix and a lysine from strand 4 appear to have favorable interaction with the phosphotyrosine ring. The peptide-binding specificity of SH2 domains is defined largely by amino acids at the +1, +2, and +3 site of the phosphotyrosine peptide.

SH3 domains. In addition to SH2 domains,

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Table 17.1.5 Protein Kinases

Protein	PDB entry	Reference
Cyclic AMP kinase	1atp	Knighton et al., 1991a. *Science* 253:407-413; Knighton et al., 1991b. *Science* 253:414-420; Olah et al., 1993. *Biochem.* 32:3649-3657
Cyclin-dependent kinase 2		De Bondt et al., 1993. *Nature* 363:592-602; Brown et al., 1995. *Structure* 3:1235-1247; Jeffrey et al, 1995. *Nature* 376:313-320
MAP kinase ERK2	1gol	Zhang et al. 1994. *Nature* 367:704-711
Twitchin kinase	S138	Hu et al., 1994. *Nature* 369:581-584
Human insulin receptor kinase	1irk	Hubbard et al, 1994. *Nature* 372:746-754
Phosphorylase kinase	1phk	Owen et al., 1995. *Structure* 3:467-482
Cyclin A	1fin	Jeffrey et al., 1995. *Nature* 376:313-320
Calcium/calmodulin–dependent kinase		Goldberg et al., 1996. *Cell* 84:875-887
Cell cycle protein CksHs1	1dks	Arvai et al., 1995. *JMB* 249:835-842

many receptor tyrosine kinases also contain SH3 domains, a conserved module of 50 to 75 amino acids. These SH3 modules are also found in adaptor proteins Grb2 and Crk, PLC-γ1, some cytoskeletal proteins like myosin 1B, fodrin, and the yeast actin-binding protein ABP-1. The function of the module is to bind proline-rich sequences on effector proteins.

The core of an SH3 domain consists of two perpendicular antiparallel β sheets folded into a β barrel–like structure. One sheet is formed with strands B, C, and D, and the other is formed with strands A and E (Fig. 17.1.8B). Much of the binding site for proline-rich peptides is formed by two loops of variable length—one between strands A and B (the so-called RT loop) and one between the strand B and C (the so-called n-Src loop).

PH domains. Each individual pleckstrin homology (PH) domain is ~100 amino acids in size. It is present in many kinases, in isoforms of phospholipase C, in GTPases, in GTPase-activating proteins, and in nucleotide-exchange factors. The function of this domain remains to be elucidated. However, many PH domain–containing proteins interact with G-protein subunits, and it has been demonstrated that a rat PLC-δ_1 forms complexes with inositol-1,4,5-trisphosphate (IP_3).

Each PH domain folds into a seven-stranded antiparallel β sandwich flanked by a C-terminal long α helix at one edge of the sandwich. Strands A, B, C, and D form one sheet of the β sandwich and strands E, F, and G form the other (Fig. 17.1.8C). The identified IP_3-binding site is located at the two loop regions, loop AB (the loop between strand A and B) and CD (the loop between strand C and D), opposite the long helix.

Protein kinase fold

A common fold shared by the catalytic core of protein kinases has emerged over the last few years. Members of this superfamily include the catalytic core of all receptor serine/threonine kinases, receptor tyrosine kinases, cyclic AMP-dependent protein kinases (cAPK), phosphorylase kinases, myosin light-chain kinases, calcium/calmodulin-dependent kinases, mitogen-activated protein kinases (MAPK), and cell-cycle cyclin–dependent kinases. Such proteins often constitute the central components in the regulation of signal-transduction pathways by controlling the level of phosphorylation on their protein substrates. The three-dimensional structures of several members of this superfamily have been determined (see Table 17.1.5 for references for specific proteins). Figure 17.1.8D shows the crystal structure of cAPK as determined by Knighton and coworkers (see Table 17.1.5). It displays a bilobal fold with a smaller N-terminal lobe that consists primarily of a five-stranded antiparallel β sheet and an α helix and a larger C-terminal lobe that is primarily α-helical (8 to 10 α helices and a few short β strands; Fig. 17.1.8D). The structures of both N- and C-terminal domains are well conserved among members of the family, whereas the relative domain orientation varies among family members. The fold of the N-ter-

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Table 17.1.6 Phosphatases

Protein	PDB entry	Reference
Alkaline phosphatase	1ali	Kim and Wyckoff, 1991. *JMB* 218:449-464
Phosphotyrosine protein phosphatases		
Low-molecular-weight	1phr	Su et al., 1994. *Nature* 370:575-578
High-molecular-weight	2hnq	Barford et al., 1994. *Science* 263:1397-1403
Dual-specificity (VHR)	1vhr	Yuvaniyama et al., 1996. *Science* 272:1328-1331
Zinc/iron phosphatases		
Purple acid phosphatase	1kbp	Strater et al., 1995. *Science* 268:1489-1492
Protein serine/threonine phosphatase I	1fjm	Goldberg et al., 1995. *Nature* 376:745-753
Sugar phosphatase		
Fructose 1, 6-biosphastase	5fbp	Bone et al., 1992. *PNAS* 89:10031-10035

minal lobe is also observed in the structure of the human cell-cycle protein CksHs1. The active site of cAPK is located between the two lobes. It consists of a nucleotide- (e.g., ATP)-binding site with the nucleotide interacting mostly with the N-terminal lobe, metal-binding sites in the vicinity of the nucleotide phosphate, an activation loop positioned at the opening cleft between the two lobes, and a substrate-binding site that involves residues mainly from the large C-terminal α-helical domain. The enzyme exists in two conformations, an open form and a closed form that is generated by a rotation of the small N-terminal domain relative to the large C-terminal domain.

Phosphatases

The three-dimensional structures of several families of phosphatases have been determined in the past few years. They are classified as alkaline phosphatases, phosphotyrosine protein phosphatases I, phosphotyrosine protein phosphatases II, sugar phosphatases, and Zn/Fe phosphatases (see Table 17.1.6 for references for specific proteins).

Alkaline phosphatases. The overall structure of an alkaline phosphatase (AP) monomer is shown in Figure 17.1.9A. The enzyme exists as a symmetric dimer. The monomer displays a two-domain organization in which both domains are α/β/α sandwiches. The smaller domain consists of a three-stranded antiparallel β sheet sandwiched in between two α helices, one on each side. The larger domain has a central ten-stranded β sheet in the middle flanked by 13 helices of various lengths. Except for strand 9, which is antiparallel, the rest of central β sheet consists entirely of parallel strands.

Phosphotyrosine protein phosphatases (PTPs). Members of this superfamily can be further divided into subfamilies I and II. Subfamily I is also called the low-molecular-weight phosphotyrosine protein phosphatase family. The structure of this subtype is composed of a single α/β domain containing a central four-stranded parallel β sheet flanked by six helices on both sides (Fig. 17.1.9B).

The second family is the high-molecular-weight phosphotyrosine protein phosphatase family. It includes human PTP1B, *Yersinia* PTP, and a dual-specificity protein phosphatase VHR. The structure common to this family is a central five- to ten-stranded mixed β sheet that adopts a highly twisted conformation. The central β sheet is sandwiched between six α helices (Fig. 17.1.9C). Although the topology of this family of phosphatases is very different from that of the low-molecular-weight phosphatases, the active-site phosphate-binding loop of both subfamilies I and II shows a striking structural similarity, and they both feature a cysteine residue in the active site.

Zn/Fe phosphatases. Members of this fold include acid phosphatases and protein serine/threonine phosphatases. Their basic fold consists of two central mixed β sheets—each of approximately five strands—forming a distorted β sandwich (Goldberg et al., 1995). The β sandwich is flanked by seven α helices on one side and additional two to three helices together with two to three short strands on the other side.

Sugar phosphatases. The sugar phosphatase fold is shared among the families of fructose-1,6-bisphosphatase, inositol monophosphatase, and inositol polyphosphate-1-phosphatase. The molecule folds into a five-layered α/β/α/β/α sandwich (Fig. 17.1.9D). The two β sheets are five and eight-stranded, respectively, and mostly antiparallel. The location of the AMP binding site is at the end of the N-terminal α helix. The fold also remotely resembles the

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actin fold (see discussion of Proteins Involved in Cytoskeleton and Muscle Movements).

Cyclin fold

The eukaryotic cell cycle is regulated by a series of cyclin-dependent kinases (CDKs) that are activated by association with cyclins. Several different forms of cyclins—cyclin A, B, C, D, E, and H—are known to regulate separate CDKs. Conserved among these cyclins is a region of ~100 amino acids, termed the cyclin box. Two crystal structures reported recently have illustrated the basic fold that is represented by the so-called cyclin box (Brown et al., 1995; Jeffrey et al., 1995). It contains five α helices, with α3 being the central helix surrounded by the remaining four helices. Based on the structure of a cyclin A-CDK2 complex, the activation of CDK has been postulated to occur through a cyclin-induced conformational change in the PSTAIRE helix and the T loop of the kinase domain.

DNA-Binding Structural Motifs and Domains

Numerous DNA-binding domains are observed to utilize a small number of conserved substructures or structural motifs for binding DNA. In most cases, a portion of the structural motif protrudes from the domain and interacts with the major and/or minor groove of DNA, and also with the phosphate backbone, through hydrogen bonds, salt bridges, and nonpolar van der Waals interactions. By far the most prevalent interactions involve an α helix binding in the major groove. Other less common interactions include β strands or loops binding in either the major or minor groove of DNA. In a few cases, considerable distortion of B-form DNA is induced upon binding. This section will summarize some commonly observed DNA-binding structural motifs and DNA-binding domains. Table 17.1.7 contains references for specific proteins.

Helix-turn-helix motifs

The helix-turn-helix (HTH) structural motif, first observed in the crystal structure of the λ Cro protein in 1981, is one of the most common and well-characterized DNA-binding motifs, found in a very diverse group of proteins (Table 17.1.7). The HTH motif is part of a three-helix bundle in which the second and third helices (α2 and α3) of the bundle make direct contact to DNA. The third helix, or the "recognition helix" protrudes from the rest of the protein into the major groove of DNA, making the majority (but not all) of the base-specific contacts. The second helix sits above the recognition helix at an ~120° angle to it, forming a bridge across the two phosphate backbones on either side of the major groove (Fig. 17.1.10A). The N-terminal helix (α1) lies across the second helix opposite the DNA, completing the helical bundle where it forms interactions with the rest of the protein and may or may not interact with the DNA.

Currently, HTH motifs can be classified into four major categories based on their structures: prokaryotic repressor HTH (Fig. 17.1.10B), eukaryotic homeodomain HTH (Fig. 17.1.10C), the winged helix (Fig. 17.1.10D), and the PurR repressor HTH (Fig. 17.1.10E). Some structures fall into more than one category. The structures of prokaryotic repressor HTHs have served as the prototypical model for the overall HTH motif. In this particular class, the HTH motif by itself is not stable enough to fold or bind DNA, but requires the scaffolding of the rest of the protein. Furthermore, monomers often lack the DNA-binding ability, and dimerization is required for binding to DNA (Pabo and Sauer, 1992). Interestingly, outside the HTH motif of prokaryotic repressors, there is little structural consensus among these DNA-binding proteins. Their structures have been observed to vary from α/β folds to all-helical folds.

The eukaryotic homeodomain HTHs all use an N-terminal arm from helix 1 to bind DNA in the adjacent minor groove (Wolberger, 1996). Some also use a C-terminal arm for minor-groove binding as well. The additional interactions of the arm(s) enable homeodomains to bind DNA as monomers (Pabo and Sauer, 1992) although most homeodomains are observed to function as homo- or heterodimers in vivo. Four of the proteins in this class (yeast RAP1 telomere-binding protein, proto-oncogene product Myb, the paired-class homeodomains, and the Oct-1 POU domain) contain two HTH motifs connected in tandem by a polypeptide linker.

Another class of HTH proteins contains the so-called "winged-helix" motif (Fig. 17.1.10D). First characterized as a motif in the eukaryotic HNF-3/fork-head transcription factor, this motif uses one or two flexible loops (or wings) that protrude from the HTH on either side at approximately right angles to the recognition helix to provide additional DNA contacts. The more prominent wing is usually part of a small three- or four-stranded antiparallel β sheet, a distinctive feature of the winged-helix motif.

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Table 17.1.7 DNA-Binding Proteins

Protein	PBD entry	Reference
Helix-turn-helix		
Prokaryotic repressor proteins		
λ Cro	1cro	Anderson et al., 1981. *Nature* 290:754-758
E. coli CAP	1cgp	Schultz et al., 1991. *Science* 253:1001-1007
λ repressor	1lmb	Jordan and Pabo, 1988. *Science* 242:893-899
434 repressor	2or1	Aggarwal et al., 1988. *Science* 242:899-907
434 Cro	2cro	Wolberger et al., 1988. *Nature* 335:789-795
Trp repressor	1tro	Otwinowski et al., 1988. *Nature* 335:321-329
Mu Ner protein	1ner	Strzelecka et al., 1995. *Structure* 3:1087-1095
Biotin repressor	1bia	Wilson et al., 1992. *PNAS* 89:9257-9261
Tet repressor	2tct	Hinrichs et al., 1994. *Science* 264:418-420
Lac repressor	1lcc	Chuprina et al., 1993. *JMB* 234:446-462
Factor for inversion stimulation (Fis)	1fia	Kostrewa et al., 1992. *JMB* 226:209-226
Hin-related prokaryotic DNA recombinase	1hcr	Feng et al., 1994. *Science* 263:348-355
Homeodomains or homeodomain-like proteins		
MATα1/MATα2	1yrn	Li et al., 1995. *Science* 270:262-269
Even skipped		Hirsch and Aggarwal, 1995. *EMBO J.* 14:6280-6291
Engrailed	1hdd	Kissinger et al., 1990. *Cell* 63:579-590
Antennapedia	1hom	Qian et al., 1989. *Cell* 59:573-580
Oct-1 POU	1oct	Klemm et al., 1994. *Cell* 77:21-32
Paired (PAX) class	1fjl	Wilson et al., 1995. *Cell* 82:709-719
Yeast RAP-1		König et al., 1996. *Cell* 85:125-136
Myb (protooncogene product)	1mse	Ogata et al., 1994. *Cell* 79:639-648
Hin-related prokaryotic DNA recombinase	1hcr	Feng et al., 1994. *Science* 263:348-355
Winged-helix proteins		
HNF-3/fork-head transcription factor		Clark et al., 1993. *Nature* 364:412-420
Heat-shock transcription factor	2hts	Harrison et al., 1994. *Science* 263:224-227
LexA repressor	1lea	Fogh et al., 1994. *EMBO J.* 13:3936-3944
Histone H5 globular domain	1hst	Ramakrishnan et al., 1993. *Nature* 362:219-223
ETS transcription factor	1etc	Donaldson et al., 1996. *EMBO J.* 15:125-134
E. coli CAP protein	1cgp	Schultz et al., 1991. *Science* 253:1001-1007
Biotin repressor	1bia	Wilson et al., 1992. *PNAS* 89:9257-9261
PurR repressor		
PurR	1pru	Schumacher et al., 1994. *Science* 266:763-770
MADS box domain		
Serum response factor		Pellegrini et al., 1995. *Nature* 376:490-497
Zinc-containing DNA-binding motifs		
Cys_2 His_2 Zinc fingers		
ZIF286	1zaa	Pavletich et al., 1991. *Science* 252:809-817
GLI oncogene product		Pavletich and Pabo, 1993. *Science* 261:1701-1707
Tramtrack	2drp	Fairall et al., 1993. *Nature* 366:483-487
GATA-binding motif		
GATA-1	1gat	Omichinski et al., 1993. *Science* 261:438-446

continued

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Table 17.1.7 DNA-Binding Proteins, continued

Protein	PBD entry	Reference
Zinc-containing DNA-binding motifs (continued)		
Zn_2Cys_6 binuclear cluster		
GAL-4	1d66	Marmorstein et al., 1992. *Nature* 356:408-414
Pyrimidine pathway regulator 1 (PPR1)	1pyi	Marmorstein and Harrison, 1994. *Genes & Dev.* 8:2504-2512
Nuclear hormone receptor DNA-binding domains		
Glucocorticoid receptor	1gdc	Luisi et al., 1991. *Nature* 352:497-505
Estrogen receptor	1hcq	Schwabe et al., 1993. *Cell* 75:567-578
9-*cis*-retinoic acid receptor	2nll	Rastinejad et al., 1995. *Nature* 375:203-211
Thyroid hormone receptor	2nll	Rastinejad et al., 1995. *Nature* 375:203-211
Transcription elongation factor TFIIS		
Transcription elongation factor TFIIS	1tfi	Qian et al., 1993. *Nature* 365:277-279
p53 tumor suppressor		
p53 tumor suppressor	1tsr	Cho et al., 1994. *Science* 265:346-355
Basic leucine zipper		
GCN4 homodimer	1ysa	Ellenberger et al., 1992. *Curr. Opin. Struct. Biol.* 3:12-21
c-Fos-c-Jun heterodimer	1fcs	Mark and Harrison, 1995. *Nature* 373:257-261
Helix-loop-helix		
MAX transcription factor		Ferré-D'Amaré et al., 1993. *Nature* 363:38-45
MyoD transcription activator	1mdy	Ma et al., 1994. *Cell* 77:451-459
Upstream stimulatory factor (USF)		Ferré D'Amaré et al., 1994. *EMBO J.* 13:180-189
Transcription factor E47		Ellenberger et al., 1994. *Genes & Dev.* 8:970-980
HMG box domain		
Human SRY		Werner et al., 1995. *Cell* 81:705-714
LEF-1 transcription factor	1lef	Love et al., 1995. *Nature* 376:791-795
HMG1 B domain		Weir et al., 1993. *EMBO J.* 12:1311-1319
β-sheet motif		
Ribbon-helix-helix		
Arc repressor	1par	Raumann et al., 1994. *Nature* 367:754-757
Met repressor	1cma	Somers and Phillips, 1992. *Nature* 359:387-393
Mnt repressor	1mnt	Burgering et al., 1994. *Biochem.* 33:15038-15045
Histone-like protein (HU)	1hue	Tanaka et al., 1984. Nature 310:376-381; Vis et al., 1995. *JMB* 254:692-703
TATA box–binding protein	1ytb, 1tgh	Kim et al., 1993. *Nature* 365:512-520; Kim et al., 1993. *Nature* 365:520-527.
Rel family eukaryotic transcription factor		
NF-κβ (p50)	1nfk, 1svc	Ghosh et al., 1995. *Nature* 373:303-310; Müller et al., 1995. *Nature* 373:311-317
Histone-fold proteins		
Nucleosomal core octamer	1hio	Arents et al., 1991. *PNAS* 88:10148-10152
Drosophila $TAF_{II}42$ and $TAF_{II}62$	1taf	Xie et al. 1996. *Nature* 380:316-322

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The PurR repressor of the LacI family represents a more extreme variant of the HTH motif, with three major differences from the canonical HTH. First, although the spatial orientation is consistent with the HTH, the connectivity of the three helices is completely different, so that helices 1 and 2 interact with the DNA instead of helices 2 and 3 (Fig. 17.1.10E). Second, the orientation of the HTH with respect to the DNA dyad is in the reverse direction from what is observed in the classical HTH. Thirdly, in addition to the HTH motif, a fourth helix (the "hinge helix") is present to bind the DNA in the adjacent minor groove. In a physiological dimer, the two symmetry-related hinge helices interact with each other in an antiparallel manner to pry open the minor groove with a set of "leucine levers" which forces the DNA axis to bend 45°.

MADS box domain

The MADS box family of proteins comprises many eukaryotic regulatory proteins, including human serum response factor (SRF), *Drosophila* trachea-development factor (DSRF), the METF2 myocyte-specific enhancer factors, and the MCM1 gene regulator from yeast, all of which contain a highly conserved 60-residue core consensus sequence. SRF is the first example for which a three-dimensional structure has been determined. This protein is homodimeric and consists of three layers. The first layer is a left-handed antiparallel coiled coil formed from an amphipathic helix from each monomer. The coiled coil sits above the minor groove near the DNA dyad (the binding site) in an approximately parallel orientation with respect to the minor groove making contacts with the phosphate backbone. The DNA is bent by 72° along its helical axis at this point, wrapping around the coiled coil, enabling the N-terminal portion of the two helices to fit into the major groove of the DNA. In addition, N-terminal arms from each helix extend across the phosphate backbone and bind in the minor groove of the DNA. The coiled coil is supported by a four-stranded antiparallel β sheet that lies flat across the helices opposite the DNA. The third layer consists of a small two-helix subdomain above the sheet. In a manner that is similar to other dimeric DNA-binding proteins, the two-fold symmetry axis of the dimer often coincides with the dyad axis of the DNA-binding site.

Zinc-containing DNA-binding motifs

There are at least five classes of DNA-binding proteins that use zinc coordination to form the stable core or folding template for DNA binding. These include the TFIIIA type zinc finger, the GATA-1 Cys_4 type zinc finger, the Zn_2Cys_6 binuclear cluster, the nuclear hormone receptor DNA-binding domains, the transcription elongation factor (TFIIS), and the zinc-binding loop of the p53 tumor suppressor.

Cys_2His_2 zinc fingers. The Cys_2His_2 zinc-finger structural motif (also known as the TFIIIA zinc finger) is the most common DNA-binding motif among eukaryotic transcription factors, with several thousand protein sequences identified to date (Berg and Shi, 1996). A canonical zinc-finger motif contains the consensus sequence (Tyr/Phe)–X–Cys–X_{2-4}–Cys–X_3–Phe–X_5–Leu–X_2–His–X_{3-5}–His, where X represents any amino acid. The cysteine and histidine residues serve as zinc ligands (Berg and Shi, 1996). The structure of the motif is composed of two short antiparallel β strands followed by an α helix stabilized by a small hydrophobic core and a tetrahedrally coordinated zinc atom (Fig. 17.1.11A). The N-terminal end of the α helix rests in the major groove of the DNA, where it makes base-specific contacts; the β sheet lies on the other side of the helix opposite to the DNA (Fig. 17.1.12A) to form limited contact with the phosphate backbone. Zinc finger–containing proteins generally use this motif in tandem arrays within the DNA-binding domain. Since the three-dimensional structure of a zinc finger–DNA complex was first determined for ZIF268 from mouse intermediate protein, the structures of two other protein-DNA complexes have been elucidated, including the DNA-binding domains from the product of human oncogene GLI and the *Drosophila* regulator protein tramtrack—as well as numerous structures of uncomplexed zinc fingers.

The GATA-binding motif. The NMR solution structure of the chicken erythroid transcription factor, GATA-1, complexed with its target DNA, reveals a DNA-binding structural motif distinctly different from the canonical zinc-finger structure. The consensus sequence Cys–X_2–Cys–X_{17}-Cys–X_2–Cys characterizes an entire family of regulatory proteins expressed in numerous cell types, each of which recognize a "GATA" DNA sequence. The DNA-binding domain of GATA-1 consists of two short two-stranded irregular antiparallel β sheets followed by an α helix and a long C-terminal loop (Fig. 17.1.11C). The tetrahedrally coordinated zinc is ligated by cysteines from the first β sheet and the helix. The α helix and

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the loop that connects the two β sheets are both located in the DNA major groove, and the long C-terminal tail crosses the phosphate backbone to sit in the minor groove (Fig. 17.1.12B).

The Zn_2Cys_6 binuclear cluster. A DNA-binding structural motif containing a Zn_2Cys_6 binuclear cluster has been characterized in numerous fragments of fungal transcription factors. The three-dimensional structure of fragments of two of these proteins, GAL4 and pyrimidine-pathway regulator 1 (PPR1), complexed with DNA, have been determined. These two structures reveal homodimers, each containing three regions—two N-terminal zinc-coordinated DNA-binding modules (one in each monomer), a linker region, and a short coiled coil made from two C-terminal helices that protrude away from the DNA. The DNA-binding modules are nearly identical in both proteins—consisting of two short helices, each followed by an extended strand (Fig. 17.1.11B). Six cysteines from each module tetrahedrally coordinate two zinc atoms. An internal two-fold symmetry relates the two-helix-strand zinc-coordinated motifs within each module. In both GAL4 and PPR1, the C-terminal end of the first helix from each module points into the major groove, mediating base-specific DNA contacts to the CGG triplet. Apart from the DNA-binding motifs, the structures of GAL4 and PPR1 are rather different from each other (Fig. 17.1.12C and D). The linker region in each monomer of GAL4 is completely extended, making practically no contacts with the rest of the protein, whereas this same region in PPR1 folds into a more compact antiparallel β ribbon. The GAL4 homodimer is highly symmetric with the coiled-coil dimerization region projecting perpendicularly from the DNA. The PPR1 homodimer, however, becomes very asymmetric beyond the N-terminal DNA-binding modules, leaning to one side to form a hydrophobic core.

DNA-binding domains of nuclear hormone receptors. Eukaryotic nuclear hormone receptors form a superfamily of ligand-activated transcription factors that regulate numerous cellular processes. These proteins generally consist of an N-terminal DNA-binding domain and a C-terminal hormone-binding domain responsible for transactivation. The minimal core of the N-terminal DNA-binding domain (60 to 70 residues) consists of two helices separated by a long loop and packed against each other at approximately right angles (Fig. 17.1.11D). Two zinc atoms, one at the N terminus of each helix, are each coordinated by four cysteine side chains. The first helix, also termed the recognition helix, lies in the major groove of bound DNA, making sequence-specific contacts, and other portions of the core domain make extensive contacts with the phosphate backbone on either side of the major groove. Nuclear receptors are observed to bind cooperatively to both direct and inverted DNA repeats as either homo- or heterodimers. The three-dimensional structures of the DNA-binding domains of several nuclear receptors complexed with DNA have been elucidated. These include the glucocorticoid receptor, estrogen receptor, 9-*cis*-retinoic acid receptor, and thyroid hormone receptor.

Elongation factor TFIIS. The eukaryotic transcription elongation factor TFIIS is important in enabling RNA polymerase II to read through various hindrances such as pause or termination sites, nucleosomes, and some DNA-binding proteins. The C-terminal nucleic acid–binding domain is known to bind to both single-stranded and double-stranded DNA or RNA in gel retardation assays. The solution structure of this 50-residue domain reveals a new zinc module consisting of a small, three-stranded antiparallel β sheet that coordinates a zinc atom through four cysteine side chains located within two loops at the end of the sheet (Fig. 17.1.11E).

DNA-binding domain of p53 tumor supressor. The p53 tumor suppressor is a key protein facilitating protection against many types of cancer in animals. It is estimated that up to one-half of all cancers involve genetic inactivation of p53 (Vogelstein, 1990). The recently determined crystal structure of the DNA-binding domain of human p53 reveals an unusual fold with a larger protein scaffold than most other DNA-binding motifs. This domain consists of a sandwich of two highly twisted antiparallel β sheets packed face-to-face with a jelly-roll topology, two small helices, and a bound zinc atom at one end (Fig. 17.1.13A). Helix 2 and loop L1 bind in the major groove using a structural motif referred to as the a loop-sheet-helix, in which the loop and helix are stabilized by an intervening three-stranded β sheet. A longer loop (L3) interacts with the minor groove and is stabilized by a zinc atom that coordinates both the loop and a neighboring helix (H1) through three cysteines and a histidine. The zinc atom has been implicated in being critical for structural integrity of the domain and DNA binding.

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Basic region leucine zippers

The three-dimensional structures of the basic region leucine zipper (bZIP) domains of both the GCN4 homodimer and the c-Fos-c-Jun heterodimer, each complexed with DNA, have now been elucidated by crystallography. The bZIP eukaryotic transcription factors have relatively simple structures consisting of two long parallel α helices that dimerize via a coiled-coil leucine-zipper motif at their C-terminal end and spread apart like partially opened scissors at the N-terminal end, gripping the DNA between them (Fig. 17.1.13B). The primary sequence of leucine zippers contains heptad repeats (*a–b–c–d–e–f–g*) with hydrophobic residues at position *a*, leucine at position *d*, and charged or hydrophilic residues at the other positions (Ellenberger, 1994). Residues at positions *a* and *d* from each helix form extensive hydrophobic interactions at the core of the dimer interface, whereas charged side chains at positions *e* and *g* form inter- and intrahelical salt bridges at the edge of the interface. The N-terminal, primarily basic ends of the helices lie in the major groove on either side of the DNA, contacting base-pair edges and the phosphate backbone.

Helix-loop-helix motif

The helix-loop-helix (HLH) structural motif is similar to the bZIP domain in both its DNA-binding and dimerization strategies. As with bZIP proteins, this structural motif binds DNA as a homo- or heterodimer. Each monomer consists of two long α helices connected by a loop of variable length (5 to 23 residues) and composition, forming a left-handed parallel four-helix bundle in the dimer (Fig. 17.1.13C). The first helix in each monomer binds DNA with its N-terminal basic region sitting in the major groove on opposite sides of the DNA helix. Dimer contacts are made through the C-terminal portion of the first helix and a coiled coil formed by the second helix of each monomer. In some members of the HLH family, the coiled-coil contacts are formed by a leucine zipper; in other HLH proteins the second helix is shorter and the coiled coil is stabilized primarily through less regular hydrophobic contacts. In both cases, the dimer is required for DNA binding, and DNA bound to this motif is primarily B-form DNA, with very little distortion of the DNA observed. Examples of three-dimensional structures of HLH proteins include the eukaryotic transcription factor Max, the MyoD transcription activator, the HLH region of the upstream stimulatory factor (USF), and the E47 regulatory protein.

HMG box domains

DNA-binding proteins containing the high-mobility group (HMG) box form a diverse superfamily of eukaryotic regulatory proteins including various transcription factors, components of chromatin, products of fungal mating-type genes, and the mammalian sex-determining gene product SRY (Laudet et al., 1993). The HMG consensus box consists of ~80 residues with an average conservation between boxes of 25% amino acid identity. HMG box proteins fall into two subfamilies. The first subfamily is observed in all cell types, contains multiple HMG box domains, and tends to bind DNA with little specificity. The second subfamily is found in fewer cell types, contains only one HMG box domain, and binds DNA specifically. The recent NMR solution structures of the HMG domains in two proteins of the second subfamily, complexed with DNA, and the solution structures of uncomplexed HMG domains from the first subfamily reveal a three-helix L-shaped structure that binds DNA exclusively in the minor groove and induces profound structural changes in bound DNA (Fig. 17.1.13D). Helices 1 and 2 are antiparallel to each other (with an acute angle between them) forming the base of the L. Helix 3, the longest helix, is almost perpendicular to helix 2 and forms the vertical arm of the L. All three helices participate in forming a concave surface for binding the minor groove and phosphate backbone of the target DNA and in causing severe DNA unwinding and bending away from the major groove.

DNA-binding proteins that use β-sheet motifs

The vast majority of DNA-binding proteins structurally characterized to date are observed to interact with DNA through helical structures. However, three different families of DNA-binding proteins have been discovered in which β-sheet structures are used as the primary contacts for protein-DNA interactions.

The ribbon-helix-helix motif. The first family includes the three prokaryotic repressor proteins, Arc, MetJ, and Mnt, all of which contain the ribbon-helix-helix structural motif. All three proteins bind DNA as a dimer of dimers (homotetramers) and all have homologous structures in which the homodimer consists of a small helical bundle with a symmetric double-stranded antiparallel β sheet (β ribbon) on the surface, which can fit specifically into the major groove of DNA (Fig. 17.1.14A). In the three-dimensional structures of complexes with DNA, additional contacts are also made

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through helix and loop residues, but base-specific contacts to DNA are mediated exclusively through side chains of residues in the β ribbon. Binding of the tetramers to the two tandem operator sites is cooperative and occurs with limited distortion of B-form DNA.

The histone-like HU family. A second family of proteins that use β ribbons to bind DNA is the highly conserved histone-like HU protein family. These are prokaryotic dimeric proteins that bind DNA nonspecifically to form nucleosome-like particles containing negatively supercoiled DNA. Both crystal and NMR solution structures of a 19-kDa uncomplexed homodimeric HU protein (also known as DNA-binding protein II) from *Bacillus stearothermophilus* reveal a V-shaped protein consisting of a six-helix bundle with two three-stranded curved, antiparallel β sheets extending out from the surface to form an open cradle (Fig. 17.1.14B). Although there are no three-dimensional structures available for HU protein complexed with DNA, the structure of the homodimer can be modeled to bind DNA with two strands from each β sheet fitting into either the major or the minor groove on opposite sides of the DNA. In either case, the minor groove would be expected to widen (narrowing the major groove), thus distorting B-form DNA. Protection experiments indicate that the β ribbons bind the minor groove.

TATA-box binding protein. The third family contains only one member so far, the TATA-box binding protein (TBP), a component of the class II general transcription initiation factor TFIID, which is required for preinitiation complex formation with RNA polymerase II. TBP consists of a C-terminal region of ~180 residues, which is highly conserved across species, and a variable-length N-terminal region. The crystal structure of TBP from *Arabidopsis thaliana* (which has only a 19-residue N-terminal region) bound to the TATA-box DNA consensus sequence reveals a quasi-symmetric α/β structure composed of a curved 10-stranded β sheet with four α helices sitting on the convex side of the sheet with an overall shape resembling a saddle (Fig. 17.1.14C). Two distinct loops project away from the protein on each end of the β sheet, forming "stirrups" for the saddle. DNA containing the TATA-box consensus sequence binds to the concave surface formed by the eight central strands of the β sheet with an induced-fit mechanism, resulting in remarkably distorted DNA that is partially unwound and bent almost 90°. TBP has marked intramolecular pseudosymmetry, with an approximate two-fold axis passing through the center of the protein perpendicular to the β sheet. The primary sequence of TBP can be divided into two long repeats (89 to 90 residues each) related by 30% sequence identity. The three-dimensional structure of each half is topologically identical with a root-mean-square (rms) deviation between equivalent Cα positions of 1.1 Å.

Rel family of transcription factors

The "Rel family" of eukaryotic transcription factors is involved in mediating cellular defense responses to stress, injury, and external pathogens in vertebrates. Members of this class are defined by a 300-residue Rel homology region (RHR) and can be divided into two subfamilies: dimeric (homo- and heterodimers) and monomeric Rel proteins. The crystal structures of both human and mouse homodimers of Nf-κB (p50), bound to duplex DNA, have recently been determined independently by two groups. The p50/p50 complex differs from most other DNA-binding proteins in that protein-DNA contacts are mediated primarily through loop regions rather than α helices or β strands. The p50 protein is composed of two Ig-like domains connected by a flexible hinge region. The homodimer/DNA structure has been described as resembling a butterfly with a DNA torso and protein wings (Fig. 17.1.14D). Domain I, the DNA-binding domain, resembles the β sandwich of an Ig constant domain with the addition of a large insert between strands E and F of the Ig fold, which contains two antiparallel helices and an extension of strand E (see discussion of Immunoglobulins and the Immunoglobulin-Like Fold). This insert is a distinctive feature of the Rel-family proteins and is variable from protein to protein, suggesting a possible role in binding other transcription factors. The second domain very closely resembles an Ig variable domain minus β strands C″ and D. Both domains are oriented so that the loops at one end of the β sandwich interact with DNA, providing a continuous DNA-binding surface and inducing practically no distortion of the B-form DNA. The two N-terminal domains from each monomer provide the specific DNA contacts, and the C-terminal domains mediate dimerization contacts as well as nonspecific DNA contacts with the phosphate backbone. Of the five loops from each monomer that interact with DNA, the majority of base-specific contacts are made by the "recognition" loop connecting the first two β strands in domain I, which binds in the major groove. The other loops interact mainly with the DNA phosphate backbone.

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Histone fold

A unique fold shared by the H2A, H2B, H3, and H4 subunits of the octameric histone core of nucleosome was first described by Arents and coworkers (Table 17.1.7) as the histone fold. Recently, the determination of crystal structures of transcription factors (TAFs), particularly the amino-terminal fragments of $TAF_{II}42$ and $TAF_{II}62$ from *Drosophila*, has demonstrated another example of the histone fold. The individual polypeptides display a helix-loop-helix structure, with a central long α helix flanked on each side by a short α helix (Fig. 17.1.14E). Both the histone core subunits, H3 and H4, and the two TAF fragments, $TAF_{II}42$ and $TAF_{II}62$, form similar heterodimeric and heterotetrameric structures. The function of histones in all eukaryotic cells is to provide a scaffold for nuclear DNA to wrap around into a highly compacted nucleosome. It has been postulated that a similar TAF octameric organization is involved in transcription activation (Goldberg et al., 1995).

RNA-Binding Structural Motifs and Domains

Relative to DNA, RNA is capable of folding into a more diverse array of structural motifs. In addition to the standard A-form double helix, irregularities such as bulges, hairpin loops, helix unwinding, noncanonical base-pairing, and unstacked bases are all common in RNA structures. This results in RNA-binding protein structural motifs that are designed to interact with various perturbations of the RNA A-form helix, instead of focusing primarily on major- and minor-groove interactions as observed in DNA-binding proteins. To date, the three-dimensional structures of more than 20 RNA-binding proteins or domains have been determined, five of them including RNA in complex with the protein (see Table 17.1.8 for references for specific proteins).

The known structures of RNA-binding proteins can be divided into several classes of small RNA-binding modules (RNP, OB-type domains, dsRBD, KH domains, zinc fingers, and the four-helix bundle of Rop) as well as a category of much larger domains and oligomers that bind RNA (MS2 phage-coat protein, trp RNA-binding attenuation protein, elongation factor Tu, and tRNA synthetases).

RNP domain

The RNP domain is the most widely observed and well-characterized RNA-binding domain (Burd and Dreyfuss, 1994). It is identified in amino acid sequences by two highly conserved consensus sequences, referred to as RNP1 and RNP2, within a more weakly conserved region of ~80 residues. This domain has been characterized in sequences of more than 200 proteins that interact with RNA (Nagai, 1996). The three-dimensional structure of two RNP domains have been determined (U1A splicosomal protein and the RNA-binding domain of hnRNP C), revealing a four-stranded antiparallel β sheet with two α helices laying across one face with a βαββαβ topology. The crystal structure of the RNA-binding fragment of the U1A splicosomal protein, complexed with a 21-nucleotide RNA hairpin, shows that the RNA binds as an open structure to the exposed β sheet surface opposite the helices (Fig. 17.1.15A). Two loops from one end of the β sheet protrude through the middle of the RNA loop, positioning and stabilizing the RNA structure on the sheet and preventing base pairing. This enables sequence-specific interactions to occur between the bases and both main-chain and side-chain atoms on the β sheet. Although it has no sequence homology with RNP proteins, the ribosomal protein S6 has been observed to have the same fold with a topology identical to the U1A protein (Fig. 17.1.15B). Similar α/β folding patterns have also been observed in the structures of other ribosomal proteins such as L6, L7/L12, and L30. The bacteriophage T4 regA translational regulator protein also contains two regions that have sequence homology to the RNP1 and RNP2 sequence motifs. This protein specifically represses the translation of 35 early T4 mRNAs. The 1.9-Å crystal structure of T4 regA consists of two antiparallel β sheets (three and four strands respectively) and four helices (Fig. 17.1.15C). The larger β sheet contains the RNP1 and RNP2 sequence motifs in two adjacent β strands exposed to solvent, and superficially resembles the RNA-binding β sheet in the U1A protein.

OB fold

The cold-shock proteins CspA and CspB, from *E. coli* and *Bacillus subtilis* respectively, are implicated in binding both RNA and single-stranded DNA. Each contains a region that has sequence homology with a canonical RNP domain. The structures of these proteins, however, consist of a five-stranded antiparallel β barrel known as the oligonucleotide/oligosaccharide (OB) fold (Fig. 17.1.16A and B; Murzin, 1993). The RNP sequence motifs reside in two adjacent β strands, suggesting a possible RNA-

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Table 17.1.8 RNA-Binding Proteins

Protein	PDB entry	Reference
RNP domain		
U1A splicosome	1urn	Oubridge et al., 1994. *Nature* 372:432-458
hnRNP C		Wittekind et al., 1992. *Biochem.* 31:6254-6265
Ribosomal protein S6	1ris	Lindahl et al., 1994. *EMBO J.* 13:1249-1254
T4 regA translational regulator	1reg	Kang et al., 1995. *Science* 268:1170-1173
OB-fold		
B. subtilis major cold-shock protein (CspB)	1csp, 1nmg	Schindelin et al., 1993. *Nature* 364:164-168; Schnuchel et al., 1993. *Nature* 364:169-171
E. coli major cold-shock protein (CspA)	1mef, 1mjc	Newkirk et al., 1994. *PNAS* 91:5114-5118; Schindelin et al., 1994. *PNAS* 91:5119-5123
Ribosomal protein L14	1whi	Davies et al., 1996. *Structure* 4:55-66
Ribosomal protein S17	1rip	Golden et al., 1993. *Biochem.* 32:12812-12820
N-terminal domain of lysyl and aspartyl tRNA synthetases	1asz, 1lyl	Cavarelli et al., 1993. *Nature* 362:181-184; Onesti et al., 1995. *Structure* 3:163-176
KH domain		
Vigilin	1vih	Morelli et al., 1995. *FEBS Lett.* 358:193-198
dsRNA-binding domain		
E. coli RNase III		Kharrat et al., 1995. *EMBO J.* 14:3572-3584
Drosophila staufen protein	1stu	Bycroft et al., 1995. *EMBO J.* 14:3563-3571
N-terminal domain of ribosomal protein s5	1pkp	Ramakrishnan and White, 1992. *Nature* 358:768-771
Retroviral CCHC zinc fingers		
NCp7 complex with dACGCC	1aaf	South and Summers, 1993. *Protein Sci.* 2:3-19
NCp10		Demene et al., 1994. *J. Biomol. NMR* 4:153-170
Four-helix bundle RNA-binding protein		
E. coli ROP	1rop	Predki et al., 1995. *Cell* 80:41-50
Other RNA-binding proteins		
MS2 phage coat protein	1mst	Valegård et al., 1994. *Nature* 371:623-626
trp RNA-binding attenuation protein (TRAP)	1wap	Anston et al., 1995. *Nature* 374:693-700
Elongation factor-Tu (EF-Tu)	1ttt	Nissen et al., 1995. *Science* 270:1464-1472
Aminoacyl tRNA synthtases		
Class I		
Met tRNA synthetase		Brunie et al., 1990. *JMB* 216:411-424
Glutaminyl tRNA synthetase	1gtr	Rould et al., 1991. *Nature* 352:213-218
Tyrosyl tRNA synthetase	1tyb	Irwin et al., 1976. *JMB* 105: 577-586
Tryptophanyl tRNA synthetase		Doublie et al., 1995. *Structure* 3:17-31
Glutamyl tRNA synthetase	1gln	Nureki et al., 1995. *Science* 267:1958-1965
Class II		
Histidyl tRNA synthetase	1htt	Arnez et al., 1995. *EMBO J.* 14:4143-4155
Glycyl tRNA synthetase		Logan et al., 1995. *EMBO J.* 14:4156-4167
Seryl tRNA synthetase	1ser	Biou et al., 1994. *Science* 263:1404-1410
Aspartyl tRNA synthetase	1asz	Cavarelli et al., 1993. *Nature* 362:181-184
Phenylalanyl tRNA synthetase		Mosyak et al., 1995. *Nature Struct. Biol.* 2:537-547
Lysyl tRNA synthetase	1lyl	Onesti et al., 1995. *Structure* 3:163-176

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binding site. A similar β-barrel structure is also observed in the ribosomal proteins S17 and L14 and in the N-terminal anticodon-binding domains of both lysyl- and aspartyl-tRNA synthetases (Fig. 17.1.16C, D, and E).

KH and dsRBP domains

Two other RNA-binding domains, the K homology (KH) domain and the double-stranded RNA-binding domain (dsRBD) have structures that are similar in terms of general orientation of secondary structures (though not topology) to the structure of the RNP domain. As with the RNP domain, these domains can be identified by a short consensus sequence. The three-dimensional structures for two dsRBD-containing proteins—*E. coli* RNase III and *Drosophila* staufen protein (Fig. 17.1.17A)—and one KH motif-containing protein—human vigilin (Fig. 17.1.17C)—contain a three-stranded antiparallel β sheet with two or three helices packed against one face to form an α/β structure. Despite the superficial similarities in structure, the RNP, dsRBD, and KH domain RNA-binding structural motifs all appear to bind RNA by distinctly different modes. The dsRBD motif is postulated to interact with double-stranded RNA through the end loops of the β sheet, the N-terminal end of the last helix, and a portion of one face of the β sheet. The KH domain has been suggested to bind single-stranded RNA via the helical side of the domain (Gibson et al., 1993). The N-terminal domain of ribosomal protein S5 (Fig. 17.1.17B) from *Bacillus stearothermophilus* shares a number of conserved residues with dsRBD of staufen protein, in addition to a similar topology, suggesting it may bind RNA in the same way as a dsRBD motif.

Retroviral CCHC zinc fingers

An RNA-binding structural motif similar to the DNA-binding Cys_2His_2 zinc finger (see discussion of DNA-Binding Structural Motifs and Domains) has recently been characterized by ^1H NMR in the retroviral nucleocapsid proteins NCp7 from HIV-1 and NCp10 from Moloney murine leukemia virus. These well-conserved nucleocapsid zinc fingers use a Cys-X_2-Cys-X_4-His-X_4-Cys (CCHC) sequence motif to bind zinc, which folds into a structure that superficially resembles the classic DNA-binding Cys_2His_2 zinc finger: a small loop followed by a short one-turn helix with the zinc atom sandwiched in the middle. The results from NMR and modeling studies of a NCp7 CCHC zinc finger with single-stranded DNA indicates it binds single-stranded RNA or DNA along a hydrophobic face of the finger in a manner very different from the binding of double-stranded DNA Cys_2His_2 zinc fingers (South and Summers, 1993).

Four-helix-bundle RNA-binding protein

The RNA-binding protein Rop, from an *E. coli* plasmid, helps regulate plasmid copy number by facilitating sense-antisense RNA pairing. This protein functions as a homodimeric four-helix bundle structure comprised of two helix-turn-helix monomers (Fig.17.1.18A). Rop has no structural or sequence homology to any other known RNA-binding proteins. The results of mutation studies combined with binding assays provide strong evidence that RNA binding occurs along one face of the bundle formed by helices 1 and 1′ (Predki et al., 1995).

Large RNA-binding structures

MS2 phage coat protein. One hundred and eighty copies of MS2 phage coat protein are assembled to form the icosahedral viral capsid of the RNA bacteriophage MS2. Although composed of the same amino acid sequence, the 129-residue MS2 subunits are present in the viral capsid in three structurally similar, but not identical, conformations referred to as A, B and C. Icosahedral symmetry (see discussion of Large Multisubunit Proteins) results in the formation of AB and CC dimers arranged in the capsid as trimers of dimers (Fig. 17.1.18B). The dimers each consist of a ten-stranded antiparallel β sheet with two small β hairpins and four helices packed against one face of the sheet. In addition to its structural role, this protein also prevents translation of viral replicase by binding to replicase mRNA. A 19-nucleotide RNA hairpin of the Shine-Dalgarno sequence for viral replicase, diffused into crystals of empty phage capsid, was observed crystallographically to bind specifically to AB dimers of MS2 coat protein. Crystal symmetry precluded crystallographic analysis of any RNA bound to the CC dimer. The RNA bound to AB interacts as a hairpin structure lying as a rigid crescent against the face of the β sheet opposite the helices, and causing very little change in the protein structure relative to the unbound form of MS2.

Trp RNA-binding attenuation protein (TRAP). TRAP from *Bacillus subtilis* binds to mRNA for the tryptophan (*trpEDCFBA*) operon in the presence of tryptophan, inducing the formation of a transcription-terminator structure and thereby halting expression of the op-

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eron as tryptophan concentrations increase. The 1.8-Å crystal structure of TRAP in its activated form, bound to tryptophan, reveals a doughnut-shaped eleven-subunit oligomer containing 56% β sheet (Fig.17.1.18C). Each 75-residue subunit is composed of both a three-stranded and a four-stranded β sheet, packed face-to-face against one another. Tryptophan binds at one end of the interface between the two sheets in each subunit towards the center of the wheel. Adjacent three- and four-stranded β sheets from neighboring subunits hydrogen bond to each other to form eleven seven-stranded β sheets, each tilted in a way that resembles the blades of a turbine. Although the RNA-binding site is not known, mutation analysis of both RNA and TRAP suggest that RNA containing eleven (G/U)AG elements forms a matching circle that binds to one side of the β-sheet wheel.

Elongation factor Tu (EF-Tu). EF-Tu is one of several elongation factors that participate in the translation of mRNA into a protein sequence on the ribosome. The crystal structure of EF-Tu from *Thermus aquaticus* in a ternary complex with Phe-tRNA^{Phe} and a GTP analog shows a three-domain structure in which all three domains interact with tRNA. The N-terminal domain 1 is a structurally conserved G-protein domain (a type of mononucleotide-binding fold; see discussion of Nucleotide-Binding Proteins) which binds guanine nucleotide. Domains 2 and 3 are seven- and six-stranded β barrels, respectively. The 3′ CCA–Phe end of the tRNA binds in a cleft at the interface between domains 1 and 2, whereas the 5′ end of the tRNA binds at the interface of all three domains. The T stem of the tRNA interacts primarily with the β barrel in domain 3. Except for the terminal Phe side chain and terminal adenine, both at the 3′ end, EF-Tu interacts primarily with the ribose moieties and the phosphate backbone of the tRNA.

Aminoacyl-tRNA synthetases (aaRS). AaRS enzymes are ATP-requiring enzymes that ligate amino acids to their cognate tRNAs during translation of RNA. The aaRS family of enzymes is remarkably diverse in terms of sequence, size, and three-dimensional structure. On the basis of sequence, structural analysis, and catalytic properties, aaRS enzymes can be divided into two classes, each comprising ten members (Eriani et al., 1990; Carter, 1993). To date, the three-dimensional structures of eleven aaRS enzymes have been determined from various organisms (GluRS, GlnRS, MetRS, TyrRS, and TrpRS—from class I—and AspRS, GlyRS, HisRS, LysRS, SerRS, and PheRS—from class II), three of them complexed with their cognate tRNA (AspRS, GlnRS, and SerRS; Cusack, 1995).

Class I aaRS enzymes all share a classical dinucleotide-binding or Rossman fold (see discussion of Nucleotide-Binding Proteins) in the catalytic domain, and generally acylate the 2′ hydroxyl of the terminal ribose. The two sequence motifs, His–Ile–Gly–His and Lys–Met–Ser–Lys–Ser, are both present in all class I enzymes in regions that bind ATP. Class I enzymes are also single-chain polypeptides, except for MetRS, TyrRS, and TrpRS, which are homodimers. The ten class I aaRS enzymes are divided into three subclasses: Ia, Ib, and Ic (Cusack, 1995).

Only one three-dimensional structure has been determined for class Ia aaRS: the structure of a tryptic fragment of a MetRS monomer missing the C-terminal 125 amino acids. This structure consists of an N-terminal catalytic domain containing the dinucleotide-binding fold with one bound zinc atom and a C-terminal wedge-shaped eight-helix bundle. Two residues (Lys465 and Trp461) in the portion of the helical domain most distal to the catalytic domain have been implicated in tRNA^{Met} anticodon loop recognition.

The two known class Ib structures (GlnRS and GluRS) are each comprised of four domains: an N-terminal catalytic domain, a smaller α/β domain inserted into the catalytic domain sequence, and two C-terminal domains proposed to interact with distal portions of the tRNA (Fig. 17.1.19A and B). In GluRS, the two C-terminal domains are both α-helical bundles, but in GluRS, they are each distorted β-barrel domains.

The two structures of the class Ic TyrRS and TrpRS are very similar to one another, each containing an α-helical noncatalytic domain and a catalytic domain. An N-terminal helical region at one end of the catalytic domain provides a dimer interface, and the C-terminal helical domain is proposed to bind tRNA with the canonical Rossman fold sandwiched in the middle (Fig. 17.1.19C). In both cases, the cognate tRNA is believed to bind across both monomers in the elongated dimer structure (Cusack, 1995).

Class II aaRS enzymes have a seven-stranded antiparallel β sheet instead of a dinucleotide-binding fold in the catalytic domain, and primarily acylate the 3′ hydroxyl of the terminal ribose (except for PheRS, which acylates the 2′ hydroxyl). The amino acid se-

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quences of class II enzymes are identified by three different sequence motifs within the catalytic domain. The first sequence motif is involved in homodimerization and the other two participate in catalysis. Seven class II enzymes are homodimers, two are $\alpha_2\beta_2$ heterotetramers (GlyRS from *E. coli* and PheRS) and one is a homotetramer (AlaRS). The ten class II aaRS enzymes are further divided into three subclasses—IIa, IIb, and IIc—according to sequence homology and quaternary structure, and differing most in the noncatalytic domains (Cusack, 1995). The IIa and IIb aaRS enzymes consist of two primary domains, a catalytic domain and an N- or C-terminal tRNA-binding domain—both of which are involved in tRNA recognition—as well as smaller variable domains inserted into the catalytic domain.

To date, the crystal structures of three class IIa aaRS have been determined (HisRS, GlyRS from *T. thermophilus*, and SerRS). The HisRS and GlyRS are similar in structure, each containing the conserved class II N-terminal catalytic domain, a small insertion domain appended to the catalytic domain, and a C-terminal anticodon loop–recognition domain consisting of a five-stranded antiparallel twisted β sheet with three helices. All three domains are proposed to interact with the cognate tRNA. The structure of SerRS is quite different from HisRS and GlyRS; each monomer consists of an N-terminal antiparallel coiled-coil domain that protrudes outward to bind the variable arm and the D and TψC loops of $tRNA^{Ser}$ and a C-terminal catalytic domain (Fig. 17.1.19E). Both monomers bind a single tRNA. SerRS does not recognize the anticodon loop, but depends primarily on the three-dimensional shape of $tRNA^{Ser}$ for recognition.

The two known structures of class IIb aaRS enzymes (AspRS and LysRS) each have an N-terminal five-stranded antiparallel β-barrel domain with an OB fold and a C-terminal catalytic domain (Murzin, 1993; Cusack, 1995). The crystal structure of the yeast AspRS-$tRNA^{Asp}$ complex shows three regions of protein-RNA contact: the N-terminal β barrel interacts with the anticodon loop on the side of the major groove, the C-terminal catalytic domain contacts the acceptor stem through loop regions containing motifs 1 and 2, and a helical hinge region connecting both domains interacts with backbone of U11 and U12 (Fig. 17.1.19D).

Of the class IIc aaRS enzymes, the PheRS crystal structure is the only one determined to date. In this $\alpha_2\beta_2$ synthetase structure, the α subunit is the canonical class II catalytic module with a small α/β insertion domain. The large β subunit, however, consists of eight different domains with the α subunit embedded in the midst of them. Several conserved structural motifs are observed in this subunit, including a catalytic-like module (domains 6 and 7) which has almost the same fold as the α subunit, two domains that resemble the winged-helix DNA-binding motif (domains 1 and 5; see discussion of DNA-Binding Structural Motifs and Domains), an RNP domain that contains the canonical RNP1 and RNP2 sequence motifs (domain 8), an SH3-like domain (domain 3; see Proteins Involved in Signal Transduction Pathways), and a six-stranded β barrel similar to the tRNA anticodon loop recognition domains in AspRS, and LysRS (domain 2).

Carbohydrate-Binding Proteins

Lectins represent a structurally diverse group of proteins that bind carbohydrate specifically. They are widely distributed throughout plants and animals and even in viruses and bacterial toxins. Although often weak in binding monosaccharides, many lectins function as oligomeric proteins to increase the valency and thus the affinity of binding toward carbohydrate ligands. Despite their functional similarity, neither the mode of oligomerization (dimers, tetramers, and pentamers all have been observed) nor the mode of carbohydrate recognition are strictly conserved in members of this family. Some members require Ca^{2+} or Mn^{2+} for saccharide binding, but others are thio-dependent (Sharon, 1993; Drickamer, 1995; Rini, 1995a,b). Lectins can be divided into subtypes according to their structural homology. Four of the more frequently observed subtypes, the legume and cereal lectins in plants and the S-type and C-type lectins in animals are described here. Generally, members within each subtype share much higher degrees of structural conservation and conservation in carbohydrate recognition than do the members of different subtypes. See Table 17.1.9 for references for specific carbohydrate-binding proteins.

Legume lectins

Lectins from various leguminous plants have very homologous three-dimensional structures and share similar locations for the carbohydrate recognition site (see Table 17.1.9 for references for specific proteins). They often form dimers or tetramers and require both Ca^{2+} or Mn^{2+} ligated to each domain for carbohydrate binding. The general fold consists of a

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Table 17.1.9 Carbohydrate-Binding Proteins

Protein	PDB entry	Reference
Soybean agglutinin	1sba	Dessen et al., 1995. *Biochem.* 34:4933-4942
Wheat germ agglutinin	2wgc	Wright and Jaeger, 1993. *JMB* 232:620-638
C-type		
Mannose-binding	2msb	Weis et al., 1992. *Nature* 360:127-134
E-selectin	1esl	Graves et al., 1994. *Nature* 367:532-538
S-type		
S-lectin	1slt	Liao et al., 1994. *PNAS* 91:1428-1432
S-Lac lectin	1hlc	Lobsanov et al., 1993. *JBC* 268:27034-27038

13-stranded β sandwich formed from a six-stranded flat β sheet packed against the convex face of a seven-stranded curled β sheet. The carbohydrate-binding site is located on the outside concave portion of the seven-stranded β sheet (Fig. 17.1.20A). Although the general topology—i.e., the connectivity and strand order—is preserved in legume lectins, some members of the family do have deleted or inserted β strands or permutated termini. Lectins usually have their N- and C-termini close to each other in structure. This makes termini permutation particularly simple without disrupting the overall structure. For example, the N and C termini for Con A from jack bean are located at the respective N-terminal end of strand F and the C-terminal end of strand E of soybean agglutinin.

Cereal lectins

Wheat germ agglutinin (WGA) represents a member of plant chitin-binding lectins that are specific for both *N*-acetylglucosamine and *N*-acetylneuraminic acid (sialic acid). It functions as a homodimer of 17-kDa subunits. The X-ray crystal structure of WGA shows that each monomer contains 32 disulfide-bonded cysteines and has a tertiary structure consisting mostly of loops and a few one-turn α helices with no regular β structure (Fig. 17.1.20B).

C-type lectins

This family of animal lectins is characterized by a conserved, roughly 120-amino-acid Ca^{2+}-dependent carbohydrate-recognition domain (CRD) that is often associated with other functional domains to mediate cell adhesion and other cellular signaling events. Members of this group include endocytic glycoprotein receptors, macrophage receptors, selectins, and the soluble collectins. Each CRD consists of a set of five to seven short β strands forming two abutting β sheets that are sandwiched in between two α helices. The carbohydrate-binding site is located in loop regions at one end of the β sheet (I–G–H; Fig. 17.1.20C). Calcium ions are directly involved in the binding of saccharide.

S-type lectins

S-lectins are a family of thio-dependent, β-galactoside-binding proteins. They have been proposed to play important roles in modulating cell-cell and cell-matrix interactions (Hirabayashi and Kasai, 1993). Galectins are the best known examples of this family. They often form dimers or associate with other accessory domains. Crystal structures of galectin 1 and galectin 2 show a striking structural homology with legume lectins, with the exception of few insertion and deletion strands. Each monomer folds into a curled β sandwich with the flat sheet consisting of five β strands and the curled sheet consisting of six β strands (Fig. 17.1.20D). Although the carbohydrate binds on the concave portion of the curled sheet, in a manner similar to what is seen in legume lectins, the specific mode of saccharide recognition is not conserved between legume lectins and galectins.

Calcium-Binding Proteins

See Table 17.1.10 for references for specific calcium-binding proteins.

EF-hand calcium binding motif

EF-hand is the most commonly observed Ca^{2+}-binding motif in proteins. Today, there are at least several hundred protein sequences and ~31 subfamilies found to contain this motif (McPhalen et al., 1991; Nakayama and Kretsinger, 1994). Examples of EF-hand-containing proteins include calmodulin, troponin C, essential light-chain myosin and regulatory light-

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Table 17.1.10 Calcium-Binding Proteins

Protein	PDB entry	Reference
EF-hand motif		
Calmodulin	1cdl	Meador et al., 1992. *Science* 257:1251-1255
Chick skeletal muscle troponin C	1top	Satyshur et al., 1988. *JBC* 263:1628-1647
Turkey skeletal muscle troponin C	5tnc	Herzberg and James, 1988. *JMB* 203:761-769
Calbindin	4icb	Svensson et al., 1992. *JMB* 223:601-606
Myosin subfragment 1	1mys	Rayment et al., 1993. *Science* 261:50-58
Scallop myosin	1scm	Xie et al., 1994. *Nature* 368:306-312
Recoverin	1rec	Flaherty et al., 1993. *Cell* 75:709-716
BM-40	1bmo	Hohenester et al., 1996. *Nature Struct. Biol.* 3:67-73
Tr_{1c} fragment of skeletal muscle troponin C	1trf	Findlay et al., 1994. *JBC* 269:6773-6778
Annexins		
Annexin V	1ala	Huber et al., 1990a. *FEBS Lett.* 275:15-21, 1990b. *EMBO J.* 9:3867-3874, 1992. *JMB* 223:683-704; Bewley et al., 1993. *Biochem.* 32:3923-3929

chain myosin, parvalbumin, intestinal and sarcoplasm calcium-binding proteins, calcineurin B, recoverin, and extracellular matrix protein BM-40. There are two kinds Ca^{2+}-binding sites among the EF-hand motif proteins, the high-affinity Ca^{2+}-binding sites and the low-affinity sites. The high-affinity Ca^{2+}-binding sites appear primarily to provide the structural stability of proteins, whereas the low-affinity sites often function to regulate the active protein conformations, so they are also named regulatory sites. For example, calmodulin undergoes a conformational change upon binding of Ca^{2+} ions to the regulatory sites. Both crystal and NMR structures of a number of EF-hand proteins have been solved. A typical EF-hand consists of a helix-loop-helix arrangement with the two helices orientated roughly 90° with respect to each other and the Ca^{2+}-binding site located in the loop region (Fig. 17.1.21A). In most situations, they form pairs with the two EF-hands in a pair related approximately by a two-fold symmetry. The Ca^{2+} coordination is usually described as a pentagonal bipyramid geometry with a total of six to seven ligands, which are from the carboxylate groups of Asp, Glu, and main-chain carbonyl oxygens in the loop region. Water molecules are often found to provide one of the apical coordinations.

Annexins

Annexins are a family of calcium-dependent phospholipid-binding proteins. Unlike the classical EF-hand calcium-binding proteins, the annexins chelate calcium in an entirely different way. Each calcium-binding domain comprises five α helices—A through E (Fig. 17.1.21B). The coordination geometry is that of a pentagonal bypyramid with three ligands from the backbone carbonyl oxygen groups of the residues in AB loop (the loop between helices A and B). The three ligands are each separated by one amino acid in the sequence. Two other ligands are provided by the side-chain carboxylate of an Asp or Glu residue, located at the end of helix D, 44 amino acids downstream from the first carbonyl ligand of the AB loop. The six and seventh ligand are less conserved and can be replaced with water molecules.

Nucleotide-Binding Proteins

Nucleotide-binding folds first began to be characterized in the early 1970s (Rossman et al., 1975). It was observed that single domains with conserved folding patterns were responsible for nucleotide binding in many different proteins. Since then, this fold has been divided into four primary classes: dinucleotide-binding domains, the mononucleotide-binding fold, the mononucleotide binding fold of protein kinases (see discussion of Proteins Involved in Signal-Transduction Pathways), and the actin fold (see discussion of Proteins Involved in Cytoskeleton and Muscle Movements). However, not all nucleotide-binding proteins have these folding patterns. Proteins such as the class II aaRS enzymes (containing an antiparallel β sheet for

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Table 17.1.11 Nucleotide-Binding Proteins

Protein	PDB entry	Reference
Dinucleotide-binding fold		
Canonical dinucleotide-binding proteins		Schulz, 1992. *Curr. Opin. Struct. Biol.* 2:61-67; Lesk, 1995. *Curr. Opin. Struct. Biol.* 5:775-783
Mononucleotide-binding fold		
Adenylate kinase	3adk	Schulz et al., 1974. *Nature* 250:120-123
CheY chemotactic protein	3chy	Volz and Matsumura, 1991. *JBC* 266:15511-15519
γδ-resolvase	2rsl	Sanderson et al., 1990. *Cell* 63:1323-1329
Flavodoxin	1fx1	Watenpaugh et al., 1972. *PNAS* 69:3185-3188
Glutamyl tRNA synthetase	1gln	Nureki et al., 1995. *Science* 267:1958-1965
Glutaminyl tRNA synthetase	1gtr	Rould et al., 1991. *Nature* 352:213-218
Methionyl tRNA synthetase		Brunie et al., 1990. *JMB* 216:411-424
Tyrosyl-tRNA synthetase	1tyb	Irwin et al., 1976. *JMB* 105:577-586
Tryptophanyl tRNA synthetase		Doublie et al., 1995. *Structure* 3:17-31
Myosin subfragment 1	2mys	Rayment et al., 1993. *Science* 261:50-58
Uridylate kinase	1uky	Mueller-Dieckmann and Schulz, 1994. *JMB* 236:361-367
Guanylate kinase	1gky	Stehle and Schulz, 1990. *JMB* 211:249-254
Elongation factor Tu (EF-Tu)	1ttt	Nissen et al., 1995. *Science* 270:1464-1472
Elongation factor G (EF-G)	1efg	Czworkowski et al., 1994. *EMBO J.* 13:3661-3668
Transducin α	1tnd	Noel et al., 1993. *Nature* 366:654-663
H-ras p21	121p	Pai et al., 1990. *EMBO J.* 9:2351-2359
RecA (ATPase domain)	2reb	Story and Steitz, 1992. *Nature* 355:374-376
ADP ribosylation factor 1 (ARF-1)	1hur	Amor et al., 1994. *Nature* 372:704-708
Mitochondrial F_1-ATPase	1cow	Abrahams et al., 1994. *Nature* 370:621-628

binding ATP) and some FMN-binding proteins (TIM barrel structures), among others, have very different folds for interacting with single nucleotides. To avoid confusion, the di- and mononucleotide-binding folds discussed in this section are usually referred to in the literature as classical or canonical nucleotide-binding folds, and the dinucleotide-binding fold is sometimes referred to simply as the "Rossman fold." See Table 17.1.11 for references for specific nucleotide proteins.

Classical dinucleotide-binding fold

In general, the canonical dinucleotide-binding proteins fall into one of three groups: NAD(P)-dependent dehydrogenases and oxidoreductases, FAD-linked oxidoreductases, and FAD/NAD-linked oxidoreductases. The three-dimensional structures of >30 dinucleotide-binding, Rossman fold–containing proteins have been determined to date. Although many of these proteins are multidomain α/β structures, their dinucleotide-binding domains all have a common three-dimensional fold. These binding domains contain a central five- or six-stranded parallel β sheet connected to helices on each face through right-handed loops (Fig. 17.1.22A and B). The conserved topology for this α/β/α sandwich motif, as compiled by Schulz (1992), is illustrated in Figure 17.1.22B. In each case the dinucleotide binds across the C-terminal end of the sheet with the ADP portion interacting specifically with the central βαβ unit of this fold (β1–α1–β2). The core sequence motif (also called the fingerprint sequence) for this βαβ fold is Gly–X–Gly–X_2–Gly (where X is any amino acid) and is located in a loop between β strand 1 and helix 1. This sequence permits unusual main-chain dihedral angles in the binding loop and close contacts with ADP. The pyrophosphate moiety sits at the N-terminal end of the helix following the loop, a favorable environment for the negative charges. Another conserved feature is an aspartic acid residue (for binding the ribose moiety of NAD) or a glutamic acid residue (for binding the ribose moiety of FAD) at the C-terminal end of the second β strand of

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Table 17.1.12 Replication and Transcription Enzymes

Protein	PDB entry	Reference
DNA/RNA polymerases		
DNA polymerase β (pol-β)	1bpd	Sawaya et al., 1994. *Science* 264:1930-1935
Klenow fragment of *E. coli* DNA polymerase	1kln	Beese et al., 1993. *Science* 260:323-355
Taq DNA polymerase	1taq	Kim et al., 1995. *Nature* 376:612-616
HIV-I reverse transcriptase	1hmi	Jacobo-Molina et al., 1993. *PNAS* 90:6320-6324
T7 RNA polymerase	2rnp	Sousa et al., 1993. *Nature* 364:593-599
Moloney murine leukemia reverse transcriptase	1mml	Georgeiadis et al., 1995. *Structure* 3:879-892
DNA polymerase processivity factors		
β subunit of *E. coli* polymerase III holoenzyme	2pol	Kong et al., 1992. *Cell* 69:425-437
Proliferating cell nuclear antigen (PCNA)	1plq	Krishna et al., 1994. *Cell* 79:1233-1243
Topoisomereases		
Type I		
E. coli topoisomerase I	1ecl	Lima et al., 1994. *Nature* 367:138-145
Type II		
E. coli gyrase B		Wigley et al., 1991. *Nature* 351:624-629
Yeast topoisomerase II	1bgw	Berger et al., 1996. *Nature* 379:225-232
Polynucleotide transferases		
E. coli RNase H	2m2	Katayanagi et al., 1990. *Nature* 347:306-309; Katayanagi et al., 1992. *JMB* 223:1029-1052; Yang et al., 1990. *Science* 249:1398-1405
HIV-I reverse transcriptase RNase H	1hrh	Davies et al., 1991. *Science* 252:88-95
RuvC resolvase	1hjr	Ariyoshi et al., 1994. *Cell* 78:1063-1072
HIV-I integrase	1itg	Dyda et al., 1994. *Science* 266:1981-1986
Mu transposase	1beo	Rice and Mizuuchi, 1995. *Cell* 82:209-220

the βαβ unit. NADP-binding domains contain an arginine at this position for binding the 2′ phosphate. One additional eleven-residue sequence motif has recently been located in β strand 5 of the canonical dinucleotide-binding fold in FAD-binding domains, which includes a C-terminal aspartic acid that interacts with the ribitol moiety of FAD (Wierenga et al., 1985).

Classical mononucleotide-binding fold

First noted in the structure of adenylate kinase (Fig. 17.1.22C and D), the mononucleotide-binding fold is superficially very similar to the dinucleotide-binding fold, but has a different topology. This fold also consists of an α/β/α sandwich with a central five-stranded parallel β sheet and right-handed connections to helices (Fig. 17.1.22C and D). As in the case of the dinucleotide-binding fold, mononucleotide binds to a critical glycine-rich loop at the C-terminal end of the β sheet between β strand 1 and helix 1. This loop (also referred to as the "P loop") is much longer than the shorter dinucleotide-binding loop, enabling it to form a giant positively charged anion hole for interacting with the mononucleotide triphosphate moiety (Dreusicke and Schulz, 1986). The loop's core sequence motif, Gly–X_2–Gly–X–Gly–Lys (where X is any amino acid), has been used to identify many more mononucleotide-binding proteins, including some from the more distantly related GTP-binding proteins (also known as G–proteins) which also have a P loop (Saraste et al., 1990; Schulz, 1992; Traut, 1994). On the other hand a number of protein structures have been observed to contain

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mononucleotide-binding folds but not the canonical P-loop fingerprint sequence (e.g., CheY chemotactic protein, γδ-resolvase, flavodoxin, GluRS, GlnRS, MetRS, TyrRS, and TrpRS). Class I aaRS enzymes are observed to have their own conserved sequence motifs (His–Ile–Gly–His and Met–Ser–Lys) within the ATP-binding loops of the mononucleotide-binding fold (Traut, 1994). Other mononucleotide-binding proteins with the canonical folding pattern and whose three-dimensional structures have been determined include myosin subfragment 1, uridylate kinase, guanylate kinase, elongation factor Tu, elongation factor G, transducin α, H-ras p21, recA, ADP-ribosylation factor 1 (ARF1), and mitochondrial F_1-ATPase. It should be noted that some mononucleotide-binding proteins, such as adenylate kinase, also bind monophosphate nucleotides in a distinctly different binding site (albeit close in proximity to the classical site).

Enzymes Involved in DNA/RNA Replication and DNA Transcription

See Table 17.1.12 for references for specific proteins.

DNA/RNA polymerases

DNA and RNA polymerases are fundamentally important enzymes responsible for replication, repair, and transcription of DNA and RNA in all living organisms. The structures of these enzymes vary widely, from small, single-subunit proteins only a few tens of kilodaltons in size to large multisubunit complexes approaching 1000 kDa (Joyce and Steitz, 1994). The three-dimensional structures of the intact catalytic domains of five different nucleic acid polymerases from eukaryotic, prokaryotic, and viral organisms have been solved to date. These are the 39-kDa catalytic domain of rat DNA polymerase β (pol-β), the Klenow fragment (KF) of *Escherichia coli* DNA polymerase I, the *Taq* DNA polymerase from *Thermus aquaticus*, the HIV-1 reverse transcriptase (RT), and the bacteriophage T7 RNA polymerase (RNAP). The polymerase domains of all five structures possess a common arrangement of subdomains centered around a large DNA-binding cleft which can be loosely described as a U-shaped structure resembling a person's right hand. Using nomenclature introduced by Ollis et al. (1985), the central base of the structure is referred to as the "palm" subdomain and the two vertical walls of the U are called the "fingers" and "thumb" subdomains (Fig. 17.1.23A and B). Associated catalytic functions distinct from polymerase activity—the 3′ to 5′ exonuclease activity in KF, RNase activity in RT, the 5′ nuclease activity of pol-β and *Taq* DNA polymerase, and nascent RNA binding by RNAP—are located in separate N- or C-terminal domains.

The structures of three of the polymerase domains—KF, *Taq* DNA polymerase, and RNAP—are very similar to each other, having nearly the same topology. The RT and pol-β structures, however, are quite different. Not only are the folds of the subdomains different in RT and pol-β, but the orders of the subdomains within the primary sequence are different as well. Furthermore, RT functions as a heterodimer (p66/p51), but the other four polymerase domains function as monomers. Interestingly, the p51 subunit of RT results from the cleavage of the C-terminal RNase H domain from p66. Thus, both p51 and p66 have the structurally equivalent thumb, fingers, and palm subdomains. In addition, both RT subunits contain another α/β subdomain termed the "connection" subdomain, which bridges the RNase H and polymerase domains in the larger subunit. P66 and p51 differ dramatically in the spatial arrangement of their subdomains (Fig. 17.1.23C), resulting in two morphologically and functionally asymmetric subunits, with the polymerase activity residing only in the larger p66 subunit (Joyce and Steitz, 1994).

The three-dimensional structure of a partially active fragment of Moloney murine leukemia virus reverse transcriptase (MoMuLV RT), containing only the fingers subdomain and part of the palm, has also been determined. The fold of the MoMuLV RT fingers and palm subdomains are nearly identical to the respective subdomains in HIV-1 RT, with only minor changes in subdomain orientation.

The palm is the only structurally conserved subdomain among all five polymerases. It consists of a five- to six-stranded antiparallel β sheet with two helices packed against one face of the sheet—a structure similar to the RNP motif found in many RNA-binding proteins (see discussion of RNA-Binding Structural Motifs and Domains). A central αββα topology is conserved in all five palm subdomains. The exposed side of the sheet displays the conserved active-site aspartic acid residues. In contrast to the palm subdomain, the fingers and thumb subdomains appear to be functionally related rather than structurally analogous for the five polymerase domains. The fingers subdomain of RT (Fig. 17.1.23C) consists of an α helix

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packed against a β sheet. The fingers' other four polymerase domains (Fig. 17.1.23A and B) are predominantly helical. The fingers subdomain of pol-β has an additional 8-kDa N-terminal domain of five helices, attached to it by a proteolytically sensitive hinge region (Fig. 17.1.23A). The 8-kDa domain is proposed to fold down over bound DNA. The thumb subdomains of KF, *Taq* DNA polymerase, and RNAP all consist of a helical bundle with one or two long helices. The thumb subdomains of pol-β and RT are α/β structures, each having three α helices and one and two β strands, respectively.

Modeling and structural studies suggest that during polymerization the 5′ end of the single-stranded template DNA or RNA interacts with the fingers subdomain as it rests in the large binding cleft above the palm (Joyce and Steitz, 1994). The crystal structure of duplex DNA, bound to KF in an editing complex, shows that a DNA-induced movement of the thumb can take place in KF, producing a second DNA-binding cleft between the thumb subdomain and the exonuclease domain into which the DNA binds (Fig. 17.1.23B). Both binding clefts are connected in the complex, forming a single binding channel along one face of the polymerase with an almost 90° bend in the middle. Polymerizing DNA is thus proposed to bind along the entire length of the KF channel with the 3′ primer terminus situated at the active site (Joyce and Steitz, 1994). The position of the exonuclease domain adjacent to the binding cleft in KF enables editing to take place without dissociation of the complex. HIV-1 RT appears to bind DNA in a slightly different manner. The crystal structure of a complex of HIV-1 RT with a 19/18-base duplex DNA reveals DNA bound in the polymerase cleft between the fingers and thumb (Fig. 17.1.23C). Although there is a DNA-induced movement of the thumb towards the DNA similar to that seen in the KF complex, DNA bound to HIV-1 RT does not bend around the thumb, but instead bends 40° to 45° in a different direction, allowing it to approach the RNase H domain. Polymerase activity is non-specific, so it is not surprising that in the structures of both DNA complexes DNA-protein contacts occur primarily through the DNA phosphate backbone.

DNA polymerase processivity factors

Certain DNA polymerases, such as those involved in replication, require a highly processive polymerase complex. The characteristic of processivity is conferred upon polymerases by associated factors that clamp the polymerase onto DNA in an ATP-dependent manner in order to prevent dissociation. Three of these factors have been characterized extensively—the β subunit of *E. coli* DNA polymerase III (pol III), proliferating cell nuclear antigen (PCNA) from yeast, and gene 45 protein of T4 phage. Crystal structures have been determined for the first two of these proteins.

The three-dimensional structures of the β subunit homodimer of pol III and PCNA are each star-shaped six-fold-symmetric closed circular rings consisting of a circle of 12 α helices perpendicular to the ring plane surrounded by six antiparallel β sheets. The inside diameter of both rings is 30 to 35 Å and the outer diameters are each ~80 Å. Each ring is composed of six nearly identical domains, each of which has two-fold symmetry, consisting of two four-stranded antiparallel β sheets packed edge-to-edge at a roughly 45° angle with two antiparallel helices packed against the concave side of the domain (Fig. 17.1.24A). The β sheets of adjacent domains hydrogen bond to each other, forming six curved eight-stranded antiparallel β sheets that line the outer surface of the ring. The complete ring structure results from the 12-fold repeat of the simple topological structural motif βαβββ around the entire circle (Fig. 17.1.24B). Despite the striking structural similarity, there is only 15% sequence identity when individual domains from each protein are aligned. Sequence alignments of individual domains within the same protein give similar results. A head-to-tail dimer of the β subunit of pol III forms the entire six-fold circle; each subunit comprises three domains. The PCNA ring is a trimer with two domains per subunit. Both proteins require ATP and auxiliary proteins to be assembled around duplex DNA, and do not dissociate easily unless the DNA is linearized. In each case, the ring has a substantial net negative charge, and positively charged side chains are concentrated on the twelve α helices lining the central channel, which would allow favorable interaction with the phosphate backbone of DNA passing through the channel. In addition, the orientation of helices facing the channel is such that they are nearly perpendicular to the phosphate backbone of B-form DNA, precluding specific interaction with the grooves of DNA. These physical characteristics, as well as the size of the inner diameter (30 to 35 Å) make both proteins well-suited for encircling DNA in a

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manner that allows for unrestricted movement along a duplex DNA strand during polymerization.

Topoisomerases

Topoisomerases are essential enzymes of eukaryotes, prokaryotes, and viruses that alter the topology of double-stranded DNA. They function by transiently breaking one or two strands of DNA, passing DNA through the opening, and then resealing the break (Wigley, 1995). Topoisomerases can be divided into two primary classes: type I enzymes, which transiently cleave only one strand of DNA, and type II enzymes, which cleave both DNA strands during catalysis.

Type I enzymes are monomeric proteins that relax supercoiled DNA in an ATP-independent manner. They can also catenate and decatenate nicked double-stranded circles and form a double-stranded DNA circle from two complementary single-stranded circles. The three-dimensional structure of a 67-kDa N-terminal fragment of *E. coli* topoisomerase I has been determined to 2.2-Å resolution. This fragment cannot relax supercoiled DNA, but is capable of cleaving single-stranded DNA. The missing C-terminal 30-kDa domain contains three zinc atoms coordinated by tetracysteine arrangements, and may participate in DNA binding. The N-terminal fragment is made of four distinct domains arranged around a large central channel with a diameter of 27.5 Å (Fig. 17.1.25A). The first domain is a four-stranded parallel β sheet sandwiched between two pairs of α helices—a fold similar to the classical dinucleotide-binding fold (see discussion of Nucleotide-Binding Proteins). Domain II is an unusual-looking β sandwich composed of two long curving three-stranded antiparallel β sheets that cross each other orthogonally at one end of each sheet. A single helix lies between the sheets at one end of the sandwich. The β strands of this domain line the inside of the top half of the central channel, bearing a striking resemblance to the TATA box–binding protein (see discussion of DNA-Binding Structural Motifs and Domains). Domains III and IV are each primarily helical, except for a small β hairpin in domain IV. Domain III contains the active-site tyrosine (Tyr319) and closes one side of the central channel through an extensive interface with domains I and IV (Fig. 17.1.25A). This interface effectively buries Tyr319, suggesting that large conformational changes are required during catalysis. It has been proposed that domains II and III may move together as a rigid body away from domains I and IV using a protease-sensitive region between domains II and IV as a hinge. Such a movement would open a gate to the central channel and expose the active-site Tyr319. The size of the closed channel is large enough to accommodate double-stranded B-form DNA without steric hindrance. The inner surface of the channel has a strong positive potential suitable for interaction with DNA.

Type II enzymes are multimeric proteins with homologous amino acid sequences. The eukaryotic type II topoisomerases are homodimers, and the prokaryotic enzymes are A_2B_2 tetramers where the A and B subunits correspond, respectively, to the N- and C-terminal halves of the eukaryotic monomer. Viral enzymes can have either subunit composition. Type II topoisomerases require ATP for catalysis and are capable of either relaxing DNA or introducing supercoils. Like the type I enzymes, they can also catenate or decatenate circular DNA. The crystal structures of two type II enzymes have been determined: the 43-kDa N-terminal fragment of *E. coli* gyrase B and a 92-kDa fragment of yeast topoisomerase II that is missing the N-terminal 409 residues. The two structures are complementary to each other, and together they provide a more complete model for the function of these enzymes.

The monomer structure of the 92-kDa yeast topoisomerase II fragment reveals a modular crescent-shaped protein with five domains (Fig. 17.1.25B). Berger and coworkers (Table 17.1.12) have divided the polypeptide chain into two subfragments, A′ and B′, based on their relation to the A and B subunits of the homologous bacterial enzymes. The N-terminal subfragment B′ has two domains—a five-stranded, mostly parallel β sheet sandwiched between four helices and a three-stranded antiparallel β sheet packed against one helix. The C-terminal subfragment A′ has three domains. The first domain consists of a winged-helix motif of three helices and a three-stranded antiparallel β sheet (see discussion of DNA-Binding Structural Motifs and Domains) embedded between an N-terminal α helix, two short C-terminal α helices, and a β hairpin. The active-site tyrosine residue, Tyr783, is located in a loop of the winged helix at the bottom of a short tunnel leading to the protein surface. The second domain comprises a four-stranded antiparallel β sheet packed against two helices on one face

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and an abutting three-stranded antiparallel β sheet. The third domain of subfragment A′ consists of a six-helix bundle and a small β hairpin. Three of the helices are unusually long, causing this domain to project out from the rest of the protein. In the crystal structure, the two monomers come together to form a flat, heart-shaped dimer with a large pear-shaped central cavity 60-Å high and 55-Å wide towards the base (Fig. 17.1.25C). Although the primary dimer contacts occur at the A′-A′ interface, the B′-B′ interface involves extensive overlap of domains such that the B′ subfragment from one monomer interacts with the A′ subfragment of the other monomer. At the interface between the first two domains in subfragment A′, a 20 to 25 Å groove runs across the protein surface including the region containing Tyr783. B-form DNA can be modeled into this groove with the recognition helix of the winged-helix motif lying in the DNA major groove.

The crystal structure of topoisomerase II lacks the N-terminal ATPase domain, which is postulated to lie on top of the B′ subfragments. Fortunately, the crystal structure of the ATPase domain of the homologous *E.coli* gyrase B has been determined to 2.5-Å resolution, enabling the modeling of the entire topoisomerase II structure. The 43-kDa fragment of DNA gyrase B is an elongated molecule consisting of two α/β domains, each with a unique fold. The first domain, which contains the ATPase site, is an eight-stranded, mostly antiparallel β sheet with five α helices packed against the convex face. It binds the nonhydrolyzable ATP analog adenosine-5′- (β, γ-imido) triphosphate (ADPNP) at the center of one face of the β sheet with the phosphates coordinated by a glycine-rich loop at the N-terminus of a helix. The second domain is a four-stranded mixed β sheet surrounded by four helices, including one long C-terminal helix that protrudes away from the protein. In the gyrase B dimer, the most extensive interaction is through the N-terminal domains. A long 15-residue N-terminal arm from each monomer wraps around the other monomer, contributing to the ATP-binding site. It is interesting to note that the binding of ATP is reported to induce dimerization. The C-terminal domain forms dimer contacts only through one end of the protruding helix from each monomer. This dimer structure creates a large 20-Å wide hole between the two C-terminal domains. The C-terminal half of this hole is observed to be rich in arginine residues, suggesting possible interaction with DNA.

Polynucleotidyl transferases

The determination of the crystal structures of several RNase H molecules has revealed a distinct fold that is common to all the polynucleotidyl transferases. Currently, members of this family include RNase H, RNase H domain of retroviral reverse transcriptase, retroviral integrase, the Holiday junction resolvase, and a core fragment of bacterophage Mu transposase. The sequence homology among them is usually <15%. At the center of the structure is a five-stranded β sheet, aligned in a strand order of 3–2–1–4–5. The second strand is antiparallel to the other four strands (Fig. 17.1.26A). The number and location of the helices are less conserved than the central β sheet. Helices A and E are topologically more conserved than the other helices. They are located between strands 3 and 4 and at the C-terminal of strand 5 in sequence, primarily interacting with the central β sheet. The number of helices between strands 4 and 5 varies as well as the helices after helix E (Fig. 17.1.26B). The family can be further divided into Rnase-like structures and integrase-like structures—integrase (Fig. 17.1.26C), Mu A transposase, and RuvC—with greater structure resemblance observed between the members of each subclass. Limited structural homology, particularly among the central five-stranded β sheets, is also observed with proteins containing the actin fold, as well as the Klenow fragment.

Proteins Involved in Cytoskeleton and Muscle Movements

See Table 17.1.13 for specific references for proteins involved in cytoskeleton and muscle movement.

Actin fold

The three-dimensional structures of four ATP-binding proteins—actin (Fig. 17.1.27A), the N-terminal domain of heat-shock cognate protein (hsc70; Fig. 17.1.27B), hexokinase (Fig. 17.1.27C), and glycerol kinase (Fig. 17.1.27D)—have all been found to contain a conserved structural motif, referred to as the "actin fold" despite the absence of any significant sequence homology. This fold consists of two α/β domains connected by a hinge region with the ATP binding between them (Fig. 17.1.27A; Kabsch and Holmes, 1995). Each domain can be further divided into two subdomains. The common feature in each domain is a five-stranded β sheet (with only one antiparallel strand) surrounded by three helices (subdomains 1 and 3 according to actin notation).

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Table 17.1.13 Proteins Involved in Cytoskeleton and Muscle Movements

Protein	PDB entry	Reference
Actin fold		
Actin	1atn	Kabsch et al., 1990. *Nature* 347:37-44
70-kDa heat-shock cognate protein	1nga	Flaherty et al., 1990. *Nature* 346:623-628
Hexokinase	2yhx	Anderson et al., 1978. *JMB* 123:1-13
Glycerol kinase	2gla	Hurley et al., 1993. *Science* 259:673-677
Actin-binding proteins		
Profilin	2btf	Schutt et al., 1993. *Nature* 365:810-816
Gelsolin segment I		McLaughlin et al., 1993. *Nature* 364:685-692
Spectrin	1spc	Yan et al., 1993. *Science* 262:2027-2030

The two β sheets within each protein are connected by two conserved helices and have identical topology, suggesting a gene-duplication event. In all four proteins, ATP binds at the interface between the β sheets along their C-terminal ends. The other subdomains (2 and 4) are not as well conserved among the four proteins. In actin, hsc70, and glycerol kinase, subdomains 2 and 4 are each α/β regions positioned at the C-terminal end of the sheets of the actin fold. Subdomain 4 has identical topology in the above three proteins, but a different tertiary structure in glycerol kinase. Hexokinase has only subdomain 4 (subdomain 2 is absent), which is an α-helical bundle.

Actin-binding proteins

The assembly of actin, the major component of filament, to form filament and cortex is regulated by a number of actin-binding proteins, including profilin, gelsolin, severin, and villin. These molecules, through their association with actin, not only control cellular locomotion and cell shape but also function as signaling molecules and keep actin filament in a dynamic balance between the free monomeric form and the polymerized form of actins. For example, profilin inhibits the assembly of F-actin by sequestering the monomeric form of actin molecules, whereas gelsolin controls the actin cortex formation through fragmentation of F-actin filaments. Recently determined tertiary structures of these actin-binding proteins has revealed their folds and helped to understand their functions in filament formation.

Profilin. Both an X-ray crystal structure and a NMR solution structure of profilin show a single-domain fold with a central five-stranded antiparallel β sheet packed against four α helices, two on each side of the sheet (Fig. 17.1.27E). The β sheet is curled toward the N-terminal α helices on one side, and the five strands are connected in the order of 1–2–3–4–5. Profilin, when complexed with β actin, forms two major contacts with its third helix, the amino-terminal portion of the fourth helix, and strands 4, 5, and 6.

Gelsolin. The members of the gelsolin superfamily include gelsolin, villin, and severin. The structure of the segment 1 domain of gelsolin shows the basic fold consisting of a central five-stranded β sheet flanked by two α helices, one on each side of the sheet (Fig. 17.1.27F). The central β sheet contains mostly antiparallel strands with the exception of the last strand of the sheet, which is in parallel configuration.

Spectrin repeats. Spectrins are actin cross-linking proteins. Their associations with actin generate meshworks to support the plasma membrane and facilitate cellular interactions. The so-called spectrin repeat defines a segment of 100 to 120 amino acids, and folds into a three-helix bundle (Fig. 17.1.27G). The observed repeats form a stable domain-swapped dimer in which the third helix of each monomer is exchanged in the helix bundle.

Enzymes

See Table 17.1.14 for references for specific enzymes.

α/β hydrolase fold

A distinct protein fold, observed among several hydrolytic enzymes, has emerged in the last few years. These enzymes, including serine carboxypeptidase II, acetylcholinesterase, dienelactone hydrolase, dehalogenase, and bacterial/fungal lipases, bear little or no detectable

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Table 17.1.14 Enzymes

Protein	PDB entry	References
aß hydrolase fold		
Torpedo californica acetylcholinesterase	1ack	Sussman et al., 1991. *Science* 253:872-879
Wheat serine carboxypeptidase II	1wht	Liao, 1990. *JBC* 265:6528-6531
Pseudomonas glumae triacylglycerol lipase	1tah	Noble et al., 1993. *FEBS Lett.* 331:123-128
Haloalkane dehalogenase	2had	Franken et al., 1991. *EMBO J.* 10:1297-1303
Sialidases		
Salmonella typhimurium LT2 sialidase	1dil	Crennell et al., 1993. *PNAS* 90:9852-9856
Influenza virus neuraminidase	1nnc	White et al., 1995. *JMB* 245:623-634
Influenza B neuraminidase	1nsb	Burmeister et al., 1992. *EMBO J.* 11:49-56; Varghese and Colman, 1991. *JMB* 221:473-486
TIM-barrel fold		
Chicken muscle triose phosphate isomerase	1tim	Banner et al., 1975. *Nature* 255:609-614
Protease folds		
Serine proteases		
Trypsin-like	1hyl	Fischer et al., 1994. *Protein Sci.* 3:769-778
Subtilisins	1sup	Murzin et al., 1995. *JMB* 247:536-540
Wheat serine carboxypeptidase II	1wht	Liao, 1990. *JBC* 265:6528-6531
Cytomegalovirus protease		Qiu et al., 1996. *Nature* 383:275-279; Shieh et al., 1996. *Nature* 383:279-282; Tong et al., 1996. *Nature* 383:272-275
Cysteine proteases		
Human liver cathepsin B	1huc	Musil et al., 1991. *EMBO J.* 10:2321-2330
Gal 6 bleomycin hydrolase	1gcb	Joshua-Tor et al., 1995. *Science* 269:945-950
Interleukin-1β converting enzyme	1ice	Wilson et al., 1994. *Nature* 370:270-275
Rhinovirus 3C protease		Matthews et al., 1994. *Cell* 77:761-771
Aspartic proteases		
Retroviral proteases: HIV-1 aspartyl protease	1hpx	Navia et al., 1989. *Nature* 337:615-620
Pepsin-like proteases: Renin	1bbs	Dhanaraj et al., 1992. *Nature* 357:466-472
Metalloproteases		
Zincins		
Snake venom adamalysin II	1iag	Gomis-Rüth et al., 1994. *JMB* 239:513-544
Pseudomonas aeruginosa alkaline protease	1akl	Baumann et al., 1993. *EMBO J.* 12:3357-3364
Zinc-dependent exopeptidases		
Aeromonas proteolytica aminopeptidase	1amp	Chevrier et al., 1994. *Structure* 2:283-291
Thermactinomyces vulgaris carboxypeptidase T	1obr	Teplyakov et al., 1992. *Eur. J. Biochem.* 208:281-288

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sequence homologies among themselves and differ widely in their catalytic functions (Ollis et al., 1992). Nonetheless, they share the same structural topology and furthermore have the same active-site serine-histidine-aspartate (or glutamate) catalytic triad. The prototype of this fold consists of an eight-stranded, mostly parallel β sheet connected by one or more helices between each adjacent strand (Fig. 17.1.28A and B). The strands are in the order 1–2–4–3–5–6–7–8, with strand 2 antiparallel to the rest. Although the central eight-stranded β sheet is well conserved among these enzymes, the lengths and numbers of helices spaced in between the adjacent strands are less conserved, and their positions vary significantly from enzyme to enzyme. The length and location of the loops connecting the secondary structure elements are also highly variable among the members of this family.

Sialidases

Sialidases and influenza neuraminidases form a family of glycohydrolases that cleave terminal α-ketosidically linked sialic acids of many glycoproteins, glycolipids, and oligosaccharides. Structurally, they define a prototype of β-propeller fold with six four-stranded antiparallel β sheets arranged in a manner similar to a six-blade propeller (Fig. 17.1.28C). There is a six-fold pseudosymmetry through the center of the subunit, relating each of the six blades. The topological connections between the strands within each sheet and among the adjacent sheets are identical from blade to blade. Each β sheet displays a typical right-handed twist, with strands 1 to 4 running antiparallel from the center of the propeller to the edge. The connection between each adjacent blade is always through the fourth strand of the preceding sheet to the first strand of the connecting sheet. The active site is located near the center of the propeller. Other β-propeller structures include bacterial methylamine dehydrogenase, galactose oxidase, and G-protein trimer β subunit.

TIM barrel fold

The TIM barrel fold, also referred to as an α/β barrel or a $(\beta/\alpha)_8$ fold, is one of the most frequently observed folds in protein structures (Brändén, 1991). This fold is used by at least 49 different enzymes with very diverse functions; it received its name from trisose phosphate isomerase (TIM), the protein in which it was first discovered in 1975. The three-dimensional structure of the TIM barrel is a rather elegant-looking eight-stranded parallel β barrel surrounded by a ring of helices that are essentially antiparallel to the β strands (Fig. 17.1.29A and B). Both the β strands and the α helices are inclined to the axis of the barrel. The classical topology of this structure is an array of eight tandem up-down β-α motifs that are aligned side-by-side in a parallel manner with consecutive strand order, eventually wrapping around to form a β barrel (Fig. 17.1.29C). In some TIM-barrel structures this array is not completely regular, containing small insertions in loop regions or, as in the cases of enolase and muconate lactonizing enzyme, a helix is missing between the last two β strands. To date, all of the crystal structures in which the TIM barrel has been found have been enzymes, and in almost every case the active site is located within the loop regions at the C-terminal end of the barrel (Farber, 1993).

Protease folds

The various proteases that have been examined structurally can be grouped into four primary classes based on characteristics of the active sites: serine proteases use an essential serine residue as the active-site nucleophile; cysteine proteases require an active-site cysteine nucleophile; aspartic proteases work primarily at low pH, utilizing two essential aspartic acid side chains for catalysis; and metalloproteases all contain a critical zinc atom coordinated at the active site. These four classes can be further subdivided into two or more different protein folds, despite the fact that members of a particular class share nearly the same reaction mechanism.

Serine proteases. Nearly all serine proteases contain the well-known catalytic triad Ser–His–Asp in their active sites. The three side chains are positioned close together through tertiary structure and are hydrogen-bonded to each other in a manner that causes the serine hydroxyl oxygen to be a strong catalytic nucleophile. The exception is the cytomegalovirus protease, which uses a similar Ser–His–His catalytic triad for the same purpose. Four completely different protein folds have been observed to date in this class of proteases.

Trypsin-like serine proteases are folded into two nearly identical domains, each consisting of a six-stranded antiparallel β barrel with a Greek key topology (Fig. 17.1.29D). In many cases an α helix is associated with each domain. The active site is formed from loop regions in a cleft between the two domains. The three-dimensional structures of at least 17 different eukaryotic, prokaryotic, and viral serine pro-

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teases have been observed to utilize this fold. One type of viral cysteine protease (3C cysteine protease) also has the trypsin-like fold.

The three-dimensional structures of eight different subtilisins from prokaryotic and fungal organisms show a common core-folding pattern made up of a seven-stranded parallel β sheet (strand order 2–3–1–4–5–6–7) sandwiched between two layers of α helices (Fig. 17.1.29E; Murzin et al., 1995). The active site is located at the C-terminal end of the central β sheet, with the essential serine residue situated at the N-terminus of an α helix. Additions to this core fold in some subtilisins include a small two- to four-stranded antiparallel sheet positioned at the C-terminal end of the large β sheet and a C-terminal β ribbon hydrogen-bonded to the main β sheet and extending it by two strands.

Serine carboxypeptidases are members of a larger family of enzymes known as the α/β hydrolases. These serine proteases contain a central eleven-stranded, mixed, mostly parallel β sheet (with three more strands than the canonical α/β-hydrolase fold) surrounded by 15 helices on either side of the sheet (Fig. 17.1.29F). The β sheet has a pronounced twist of almost 180°. As in the subtilisin class of serine proteases, the active site is located at the C-terminal end of the β sheet, with the catalytic serine at the N-terminal end of a helix.

The latest structure of a serine protease from human cytomegalovirus (hCMV) adds a new fold to the serine protease class. The three-dimensional structure of this single-domain protein consists of a seven-stranded, mostly antiparallel β barrel with one Greek key motif and seven helices surrounding approximately one-half of the circumference of the barrel and both ends of the barrel. The active-site Ser–His–His triad is positioned on the outside of the barrel on the face without any helices. Human CMV protease is unique among serine proteases in that the tertiary structure of its catalytic triad contains a conserved His residue in the same position where the Asp residue is normally found in the classical Ser–His–Asp triad. This protein functions physiologically as a dimer. The crystallographic dimer observed in three separate crystal structures reveals a local two-fold axis, relating both monomers in such a way that the active sites are on opposite sides of the dimer, 36.5 Å apart. This suggests that the active sites function independently. The reason for the physiological dimer is unknown.

Cysteine proteases. To date, three different subfamilies of cysteine proteases have been characterized, each of which utilizes the sulfhydryl of a cysteine residue as the active-site nucleophile. The most well-known and studied are the papain-like cysteine proteases, which are sometimes confused with the entire cysteine protease family.

The papain-like cysteine protease subfamily can be recognized by the conserved catalytic triad Cys–His–Asn, in addition to a unique fold. Analogous to the Ser–His–Asp triad of serine proteases, this hydrogen-bonded triad is also formed through tertiary interactions resulting in a nucleophilic sulfhydryl on the cysteine. The basic papain-like fold consists of two distinct domains, designated L and R in papain nomenclature (Fig. 17.1.30A). The L domain is a small three- to four-helix bundle. The R domain is comprised of a highly curved and twisted six-stranded antiparallel β sheet, flanked by one or two helices. The active site is in a V-shaped crevice between the two domains. In a manner similar to that seen with the subtilisins and serine carboxypeptidases, the nucleophilic cysteine residue is located at the N-terminal end of an α helix.

Although small structural variations of the described fold are observed in several papain-like proteases, bleomycin hydrolase from yeast has extensive structural modifications. A small helical domain is inserted into loops in the L domain, and an additional N-terminal helical domain is also present. This cysteine protease, which cleaves the anticancer peptide bleomycin, functions as a hexamer with a large central channel containing the six active sites. It also binds to double-stranded and single-stranded DNA and represses genes of the GAL system.

The subfamily of cysteine proteases homologous to interleukin 1β–converting enzyme (ICE) is rapidly growing and is observed to be important in inflammation and mammalian apoptosis. Originally known as ICE-like proteases, this subfamily is now becoming known also as caspases. The structure of ICE (caspase-1) is a tetramer made from two heterodimers related by a two-fold rotation axis. Each heterodimer (p10/p20) contains an independent active site and is derived from a 45-kDa precursor that has been cleaved in three locations. The fold of the heterodimer consists of a six-stranded, mostly parallel β sheet sandwiched between two helices on one face and four helices on the other (Fig. 17.1.30B). The active site, formed by loops at the C-terminal end of the β sheet, contains a catalytic Cys–His dyad. The active-site cysteine is situated at the end of a β strand, instead of the N terminus of

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an α helix as in the papain subfamily. The quaternary structure of ICE is unique among known proteases and may play a role in allosteric regulation of the enzyme.

Recently another set of cysteine proteases has been characterized structurally—3C cysteine protease from picornavirus. This protease uses a Cys–His–Glu catalytic triad and surprisingly it has the same fold as the trypsin-like serine proteases.

Aspartic proteases. The aspartic proteases (also known as acid proteases) contain primarily β sheet and utilize two aspartic acid residues to catalyze proteolysis at low pH. They are divided into two groups: the two-domain pepsin-like proteases found in eukaryotes and the dimeric retroviral proteases. Both groups contain a conserved catalytic triad sequence Asp–Thr/Ser–Gly at the active site, and limited regions of sequence conservation are shared between them.

The pepsin-like proteases are comprised of two pseudosymmetric domains, each containing a partly opened, six-stranded, antiparallel distorted β barrel structure with a few short helices within the loop regions (Fig. 17.1.30C). Between the domains and away from the center of the structure, a twisted, six-stranded antiparallel β sheet lies against the surface of the protein as if to form a base on which to set the molecule. Three strands from each domain contribute to the sheet. On the side of the protein opposite the six-stranded sheet, a large cleft runs across the domain interface forming the site for the polypeptide substrate to bind. The two catalytic aspartate residues (one from each domain) are located at the bottom of the active-site cleft (Fig. 17.1.30C). A long hairpin loop from the N-terminal domain forms a flap that projects over the top of the active site, shielding the active site from solvent.

Retroviral proteases. Retroviral proteases are homodimers that cleave newly synthesized viral polyproteins into individual functional units during viral maturation. The two identical subunits are structurally very analogous to the two domains of the pepsin-like proteases. Each is comprised of a six-stranded antiparallel β barrel, a single helix located at one end of the barrel, and a two-stranded β sheet at the C-terminal end of the helix (Fig. 17.1.30D). In the dimer, the two small β sheets hydrogen bond to form a four-stranded β sheet analogous to the six-stranded sheet observed in pepsin-like proteases. Two conserved, catalytic aspartic acid residues (one from each monomer) are positioned at the bottom of the active-site cleft at the subunit interface. A flap from each subunit extends over the top of the active site in a manner resembling the flap of the pepsin-like enzymes.

Metalloproteases. There are two main superfamilies of zinc-containing metalloproteases, each of which includes proteins from both prokaryotes and eukaryotes. These are the zinc-dependent endopeptidases (also referred to as "zincins"; Bode et al., 1993) and the zinc-dependent exopeptidases.

The conserved tertiary structure of the catalytic domain of zincins consists of a five-stranded, mostly parallel β sheet with two helices packed against the concave side (Bode et al., 1993; Murzin et al., 1995; Fig. 17.1.30E). This fold is generally observed in the N-terminal domains of zincins. The conserved His–Glu–X_2–His sequence motif identifies three critical active-site residues. These three residues are in a helix situated at the edge of the β sheet, with the two histidines coordinating the catalytic zinc and the glutamate polarizing a zinc-bound water that acts as a nucleophile during catalysis. Several zincins also bind multiple Ca^{2+} ions.

Zincins comprise two distinct families known as the thermolysin-like proteases and the metzincins. The known tertiary structures of thermolysin-like proteases have a small, irregular, twisted β sheet structure at one end of the central β sheet and a C-terminal helical domain with the active site in a crevice between the two domains. A conserved glutamate residue provides the forth zinc ligand. The metzincins, which include astacins, adamalysins, matrix metalloproteases, and serralysins, have an extended concensus sequence (His–Glu–X_2–His–X_2–Gly–X_2–His), including a third zinc-coordinated histidine. The name "metzincins" comes in part from a unique structural characteristic of this family referred to as a "Met-turn," which is a methionine-containing β turn located just beneath the zinc atom (Bode et al., 1993). It is noteworthy that one metzincin, an alkaline protease of the serralysins, has a particularly unusual C-terminal domain consisting of a calcium-binding 21-strand β sandwich (also referred to as a right-handed parallel β roll; Fig. 17.1.30G).

Zinc-dependent exopeptidases share a common catalytic fold consisting of an α/β/α sandwich. The central eight-stranded β sheet (strand order 1–2–4–3–8–5–7–6) is mostly parallel (only strands 2 and 8 are antiparallel) and hosts the active site at the middle of its C-terminal end (Murzin et al., 1995; Fig. 17.1.30F). Car-

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Table 17.1.15 Electron-Transfer Proteins

Protein	PDB entry	Reference
Cytochrome P_{450}	1phc	Raag et al., 1993. *Biochem.* 32:4571-4578
Yeast iso-I-cytochrome *c*	1ycc	Louie and Brayer, 1990. *JMB* 214:527-555
Cytochrome *b5*	1cyo	Mathews et al., 1972. *CSHSQB* 36:387-395
Ferritin	1bcf	Frolow et al., 1994. *Nature Struct. Biol.* 1:453-460

boxypeptidases A, B, and T each have one active-site zinc atom pentacoordinated to two histidines, a glutamate (both oxygens are ligands), and a water molecule. The aminopeptidases in this family, however, each have two zinc atoms coordinated <3.5 Å apart at the active site. As with endopeptidases, zinc-bound water in this exopeptidase family is considered a possible nucleophilic catalyst.

Electron-Transfer Proteins

There are several different superfamilies of proteins involved in processes of electron transfer (see Table 17.1.15 for references for specific electron-transfer proteins). Most of them have a heme prosthetic group or porphyrin that is central to electron transfer coordinated in a hydrophobic pocket. However, their tertiary folds vary from the four-helix bundle fold of ferritin to the mostly helical structure of cytochrome P_{450}, with multiple α helices and some β strands, to the typical cytochrome *c* fold with the polypeptide chain organized into a series of α helices and reverse turns.

Cytochrome P450

Cytochrome P_{450} catalyzes the monooxygenation of a number of organic molecules in a heme-dependent reaction mechanism. Four different types of P_{450} have been described to date—P_{450cam}, $P_{450eryF}$, $P_{450BM-3}$, and $P_{450terp}$—named, respectively, after their substrates camphor, 6-dexoerythronolide B, C-15 fatty acid, and terpineol. The sequence homology remains relatively high within each group and relatively weak between the groups. Despite low sequence homologies (<20%) and very diverse substrate specificities, a common fold is maintained among all the P_{450} cytochromes. The molecule folds into two domains, an α and a β domain. The α domain consists of a right-handed four-helix bundle sandwiched in between two clusters of helices, a top cluster of five helices and a bottom cluster of three helices with two β strands. The β domain consists of a β barrel surrounded by three helices. The β barrel is formed by two β sheets—a two-stranded β sheet and a five-stranded β sheet—folded against each other (Fig. 17.1.31A). The heme molecule is embedded in between the two domains.

Cytochrome c domain

Cytochrome *c* is often observed as an electron-transfer domain in many redox enzymes. Members of this family include cytochrome *c*, cytochrome *c2*, *c5*, *c551*, *c553*, and *c555*. Other proteins—such as the N-terminal domain of cytochrome *cd*1–nitrite reductase, flavocytochrome *c* sulfide dehydrogenase (FCSD), and di-heme cytochrome *c* peroxidase—also contain cytochrome *c*–type domains in their structures. Most cytochrome *c* proteins contain one cytochrome *c* domain. But some enzymes, including FCSD and di-heme cytochrome *c* peroxidase, contain two cytochrome *c*–type domains. The typical cytochrome *c* fold consists of three long α helices and a pair of β strands packed in the core region, as well as two additional short helices surrounding the core (Fig. 17.1.31B). The length of the helices and strands, as well as the conformations of the loops, vary considerably among the members of the fold. The heme group is coordinated by the residues from both helices and reverse turns in the core region.

Cytochrome c3

The structure of cytochrome *c3* from *Desulfovibrio desulfuricans* consists of three or four helices (often one long helix amidst other shorter helices), a pair of β hairpins, and three or four long irregular loops. There are four heme molecules ligated to the approximately 110 residues of cytochrome *c3*. The heme coordination frame is formed by packing the long helix against the β hairpins and the loop regions (Fig. 17.1.31C).

Cytochrome b5

In addition to cytochrome *b5*, the N-terminal domain of flavocytochrome *b2* also belongs to this fold family. The structure folds into a α/β/α sandwich with a central five-stranded mixed β sheet surrounded by six short α heli-

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Table 17.1.16 Globin-Like Proteins

Protein	PDB entry	Reference
Globins		
Alcaligenes eutrophus flavohemoglobin		Ermler et al., 1995. *EMBO J.* 14:6067-6077
Oxymyoglobin	1mbd	Phillips, 1980. *JMB* 142:531-554
Human deoxyhemoglobin	1hhb	Fermi et al., 1984. *JMB* 175:159-174
Pig roundworm hemoglobin		Kloek et al., 1993. *JBC* 268:17669-17671
Sea cucumber hemoglobin	1hlm, 1hlb	Mitchell et al., 1995. *JMB* 251:421-431
Phycocyanins		
Cyanobacterial C-phycocyanin	1cpc	Shirmer et al., 1987. *JMB* 186:677-695
Colicins		
Colicin A	1col	Parker et al., 1989. *Nature* 337:93-96

ces—two helices on the concave side of the β sheet and four on the other side (Fig. 17.1.31D). The heme is coordinated by the four helices and the central two strands of the β sheet. The five strands are hydrogen-bonded in a 1–5–3–2–4 order in cytochrome *b5* and 5–3–1–2–4 order in the cytochrome *b5*–type domain of flavocytochrome *b2*.

Four-helix-bundle cytochromes

There are at least three families of cytochromes known to assume the fold of four-helix bundles: cytochrome *b562*, cytochrome *c′* and ferritin. Among them, cytochrome *b562* and cytochrome *c′* share a close resemblance, both forming up-down-up-down helical bundles with a left-handed twist. The heme is inserted between helices 1 and 4, with helix 3 forming the floor (Fig. 17.1.31E). The structure of ferritin, on the other hand, forms an up-down-down-up four-helix bundle with a left-handed twist. There is a short one-turn helix at the C terminal of the four-helix bundle. Unlike the other two, ferritin forms a dimer with only one heme molecule inserted in the interface of the two-helical bundle subunits (Fig. 17.1.31F).

Globin-Like Proteins

The helical globin-like proteins encompass three different families: globins, phycocyanins, and colicin. The core fold for all of these proteins consists of an α-helical sandwich which is simply two layers of α helices stacked against each other. Using the globin nomenclature, helices A, E, and F make up one side of the sandwich and helices B, G, and H make up the other side, with helices in each side essentially orthogonal to those in the other side (Fig. 17.1.32A). Various members of these families are all completely α-helical (with the exception of flavohemoglobin), with minor differences in helix packing, which is due to the binding of associated cofactors and insertions of helices to the basic fold (Holm and Sander, 1993; also see Table 17.1.16 for references for specific proteins).

Globins

The globin family is made up of a number of heme-containing helical proteins used specifically for oxygen storage and transport within bacteria, insects, vertebrates, and plants. Myoglobin is the smallest of these proteins, consisting of the basic globin fold with the addition of two more short helices (C and D; Fig. 17.1.32A). This monomeric protein binds oxygen for storage in muscle using a heme group located in a cleft between helices E and F. Mammalian hemoglobin is composed of four separate globin chains ($\alpha_2\beta_2$) in an approximately tetrahedral arrangement (Fig. 17.1.32B). Each chain binds a heme group in the same location and orientation as in myoglobin. The binding of oxygen induces changes in the quaternary structure of hemoglobin such that four oxygen molecules are bound in a cooperative manner, making it well-suited for oxygen transport. Other nonmammalian hemoglobins are found to have very different oligomeric states. Pig roundworm hemoglobin, for example, is an octamer of a two-domain globin fold; sea cucumber hemoglobin can be in both monomeric and dimeric states at physiological conditions. Flavohemoglobin is a rather unusual globin. Unlike the other known globin structures, flavohemoglobin is an α/β three-domain protein containing an FAD-binding domain and an NAD-binding domain as well as an N-terminal

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Table 17.1.17 Toxins

Protein	PDB entry	Reference
Small, single-subunit toxins		
Margatoxin	1mtx	Johnson et al., 1994. *Biochem.* 33:15061-15070
Androctonus australis hector toxin II		Housset et al., 1994. *JMB* 238:88-103
Snake venom neurotoxin	1nxb	Tsernoglou et al., 1978. *Molec. Pharmacol.* 14:710-716
Agelenopsis aperta venom		Reily et al., 1995. *J. Biomolec. NMR* 5:122-132
Ribosome-inactivating toxins		
Ricin A	1rtc	Katzin et al., 1991. *Proteins* 10:251-259
Ricin B	2aii	Rutenber and Robertus, 1991. *Proteins* 10:260-269
ADP ribosylation toxins		
Diphtheria toxin	1ddt	Choe et al., 1992. *Nature* 357:216-222
Pseudomonas aeruginosa exotoxin A		Allured et al., 1986. *PNAS* 83:1320-1324
E. coli enterotoxin		Sixma et al., 1991. *Nature* 351:371-377
Pertussis toxin		Stein et al., 1994. *Structure* 2:45-57
Cholera toxin	1chp	Merritt et al., 1995. *Structure* 3:561-570
Superantigen toxins		
Staphylococcus enterotoxin C2	1se2	Swaminathan et al., 1995. *Nature Struct. Biol.* 2:680-686
Staphylococcus enterotoxin B		Swaminathan et al., 1992. *Nature* 359:801-806
Toxic shock syndrome toxin I		Prasad et al., 1993. *Biochem.* 32:13761-13766

globin domain. The middle FAD-binding domain is a six-stranded antiparallel β barrel with Greek key topology, and the C-terminal NAD-binding domain is a classical dinucleotide-binding domain with a five-stranded parallel β sheet. The physiological function of flavohemoglobin remains unknown.

Phycocyanins

Phycocyanins make up part of the light-harvesting antennas in cyanobacteria and red algae. The three-dimensional structure of this protein has the six helices of the globin fold with two additional antiparallel α helices protruding from the N terminus (Fig. 17.1.32C). A phycocyanobilin cofactor is located in the same place as the heme group of the globin structures (between helices E and F).

Colicins

Colicins are a class of multidomain antibacterial toxins produced by *E. coli* that participate in killing target cells by binding to surface receptors and forming pores in the cytoplasmic membrane. The crystal structure of a C-terminal fragment of colicin A is observed to have a canonical globin fold with three additional N-terminal helices and an extra C-terminal helix (Fig. 17.1.32D).

Toxins

The structures of toxins represent a collection of diverse families of folds. Some toxins, such as scorpion toxins and snake toxins, are relatively small, contain few well-defined secondary-structure elements and are rich in disulfides. Other toxins, such as diphtheria toxin and heat-labile toxin, have well-defined secondary structures and often form complicated multidomain oligomeric organizations. Today, there are >50 toxin structures that have been determined by X-ray crystallography or NMR. Most of them belong to one of several distinct structural folds that are summarized in this chapter (see Table 17.1.17 for references for specific proteins).

ADP ribosylation toxins

Diphtheria toxin (DT), *Pseudomonas aeruginosa* exotoxin A (ETA), cholera toxin, heat-labile enterotoxin, and pertussis toxin all contain ADP ribosylation domains. The basic fold of an ADP ribosylation domain (Fig. 17.1.33A) consists of a mixed structure of seven or eight β strands and several short helices. The β strands are grouped into two β sheets that are packed against each other forming the core of this domain.

The structures of diphtheria toxin (DT) and

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Pseudomonas exotoxin A (ETA) are each organized into three domains: a catalytic ADP ribosylation domain, a transmembrane domain, and a receptor-binding domain. The sequential order of the three domains is from N- to C-terminal in DT and from C- to N- terminal in ETA. The transmembrane domain in both DT and ETA is an all-helical domain. The receptor-binding domains are both immunoglobulin-like. However, the receptor-binding domain of DT resembles an immunoglobulin V domain whereas that of ETA resembles a Con A lectin domain.

Cholera toxin, pertussis toxin, and heat-labile enterotoxin belong to a family of bacterial AB5 toxins. All of them contain an ADP ribosylation A domain and a pentameric receptor-binding B domain. The topology of B domain, (Fig. 17.1.33B) contains a pentameric ring structure of layered helices-sheets-helices. The pentameric repeat unit is an α/β/α sandwich that consists of a one-turn α-helix, a six-stranded antiparallel β-sheet, and a long α-helix.

Superantigen toxins

The three-dimensional structures of staphylococcal enterotoxins A (SEA), B (SEB), C2 (SEC2), and toxic shock syndrome toxin 1 (TSST-1) define a family of toxins that function as superantigens to T cells. They display a common fold of two-domain structure (Fig. 17.1.33C). The N-terminal domain folds with a Greek key topology and consists primarily of a five-stranded β barrel with strands in the order 1–2–3–5–4. It is also a member of the oligonucleotide/oligosaccharide-binding (OB) fold (Murzin, 1993). The C-terminal domain of these toxins folds into a modified β-grasp motif. The primary feature of this domain is a long a helix packed against a five-stranded mixed β sheet.

Ribosome-inactivating toxins

Plant cytotoxins, including ricin and abrin, are members of the A,B chain toxins. The A chains of ricin and abrin, together with gelonin, α momorcharin, α trichosanthin, and shiga toxins form the family of ribosome-inactivating protein fold (RIP fold). An example of this family is the structure of the ricin A chain (Fig. 17.1.33D). It contains mixed β sheets and several α helices.

The B chain of ricin and abrin forms a β trefoil type of structure (Fig. 17.1.33E). There are two domains of such trefoils in the B chain of each toxin. Each individual domain consists largely of irregular loops (no regular secondary structure elements) that are pseudo-three-fold related. This domain is also a carbohydrate-binding domain.

Small, single-subunit toxins

Members of the scorpion toxins are usually small proteins, containing ~50 amino acids and are bound by disulfide bonds. They include scorpion margatoxin, noxiustoxin, charybdotoxin, scyllatoxin, agitoxin, chlorootoxin, toxin I5a, kaliotoxin, and others. The basic topology consists of an N-terminal α helix packed against a three-stranded antiparallel β sheet (Fig. 17.1.33F). The fold is stabilized by three sets of disulfide bonds, connected in sequential order of Cys I–IV, II–V, III–VI. Cys II and III usually follow the CXXXC pattern, whereas Cys V and VI usually follow the CXC pattern. Some members of the family, also called long-chain scorpion toxins, have an additional loop structure at the C-terminal end and an additional disulfide bond.

Most of the snake toxins have similar structures, known as the snake toxin fold. Members with known structures include sea snake erabutoxin A and B, neurotoxin I and II, cobratoxin I and II, cardiotoxin I, II, IIB, III, and V, and bungarotoxin. Their structure has two antiparallel β sheets, an N-terminal two-stranded β sheet, and a C-terminal three-stranded β sheet (Fig. 17.1.33G). There are four disulfides with the sequential bonding of Cys I–III, II–IV, V–VI, and VII–VIII in each structure.

The family of spider toxins include ω conotoxins from sea snail and agatoxins from web spider. These are very small proteins, <40 residues in sequence, and are stabilized by cystine bridges. The structure consists of largely irregular loops with only two short β strands forming an antiparallel β sheet at the center of the fold (Fig. 17.1.33H). The three common disulfides are connected with the sequence order of Cys I–IV, II–V, and III–VI. Members of the agatoxins have one more disulfide near the C-terminal end.

Large Multisubunit Proteins

See Table 17.1.18 for references for large multisubunit proteins.

Chaperonin proteins: GroES and GroEL

Chaperonins are a class of proteins whose function is defined as assisting in catalyzing the folding of other proteins. The GroEL/GroES complex represents one of the best-studied chaperonin systems. However, the architecture of these molecules has remained undefined

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Table 17.1.18 Large Multisubunit Proteins

Protein	PDB entry	Reference
Chaperonins		
GroEL	1oel	Braig et al., 1994. *Nature* 371:578-586; Braig et al., 1995. *Nature Struct. Biol.* 2:1083-1094
GroES	1lep	Hunt et al., 1996. *Nature* 379:37-45; Mande et al., 1996. *Science* 271:203-207
Myoglobin subfragment-I	2mys	Rayment et al., 1993. *Science* 261:50-58
G-protein trimer		Lambright et al., 1996. *Nature* 379:311-319
F_1-ATPase	1cow	Abrahams et al., 1994. *Nature* 370:621-628
T. acidophilium 20S proteosome		Lowe et al., 1995. *Science* 268:533-539

until recent years. Now, with the work from several groups, the crystal structures of both GroEL and GroES have been determined. The crystal structure of an *E. coli* GroEL was determined by Braig and coworkers (Table 17.1.18) and refined to 2.8-Å resolution. That study demonstrated an overall association of the two heptameric rings of GroEL stacked in back-to-back fashion (Fig. 17.1.34A and C). Each individual protomer is composed of three distinct domains: equatorial, intermediate, and apical. The equatorial domain consists primarily of six long α helices, two short helices and three pairs of short β strands. This is also the ATPase domain of GroEL. The intermediate domain contains a three-stranded β sheet and three α helices. The apical domain consists of a mixture of five α helices and a seven-stranded β sandwich. Structurally, the equatorial domain provides most of the intersubunit contacts within each heptameric ring and all the contacts between the two back-to-back-stacked heptameric rings. The intermediate domain appears to function as a bridge between the equatorial and the apical domains. Most of the contacts between GroEL and GroES are mediated through the apical domain.

The crystal structure of GroES, published by both Hunt and coworkers and Mande and coworkers (Table 17.1.18), shows an overall heptameric dome association of the molecule. Each individual subunit folds into a five-stranded, antiparallel β barrel (Fig. 17.1.34B). The bottom flat face of the GroES dome interacts with the GroEL.

The detailed mechanism of the assembly of GroES onto GroEL is still at the stage of electron micrography and molecular modeling. There is no structure of the entire GroEL/GroES complex available today. However, combining the molecular modeling, electron-micrographic images of the complex, and the individually determined crystal structures of GroEL and GroES, several critical regions of the chaperonin assembly have been proposed.

Myosin subfragment 1

The crystal structure of a myosin subfragment 1 determined recently illustrates the overall arrangement of myosin heavy chain, regulatory light chain, and essential light chain. The heavy chain forms much of the myosin head and a long coiled-coil helix extending ~85 Å to the C terminus of the heavy chain. The myosin head consists of mostly α helices with the actin-binding site located near the end. Both the regulatory and essential light-chain subunits bind to the long coiled-coil helical tail of the heavy chain. The structure of the regulatory light chain resembles that of calmodulin, with a divalent cation-binding site in its first EF-hand region. The essential light chain consists mostly of helical elements that wrap around the heavy chain helical tail.

G-protein trimer

A recently determined crystal structure of a heterotrimeric $G_t\alpha/_i\alpha$GDP $G_t\beta\gamma$ complex reveals the mechanism of the nucleotide-dependent engagement of the α and βγ subunits and their interaction with effector molecules. The $G_t\alpha$ subunit is organized into three structural components: a Ras-like GTPase domain that confers the nucleotide-binding fold, an all α-helical domain with a central long helix surrounded by five shorter helices, and an N-terminal helix that projects away from the remainder of the $G_t\alpha$. The $G_t\beta$ subunit has an N-ter-

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minal helix followed by a seven-fold repeating WD motif that is organized into a β propeller structure. A WD repeat refers to a sequence motif of ~40 residues; it contains several conserved amino acids, including a Trp-Asp (WD) peptide near the end of the repeat. Each WD domain contains four antiparallel β strands. The so-called WD sequence corresponds to the three inner strands of a blade together with the outside (fourth) strand of the preceding blade. The $G_t\gamma$ subunit consists of two helices that pack against the β subunit.

F_1-ATPase

The F_1F_o-ATPase found in the membranes of mitochondria, chloroplasts, and bacteria is responsible for synthesizing ATP from ADP and inorganic phosphate by utilizing a proton gradient that has been generated across the membrane. The large multisubunit ATPase complex is composed of an integral membrane stalk, referred to as F_o, and a globular head region located at one end of the stalk, called F_1, which can be easily dissociated in a soluble form. Attempts to determine the crystal structure of the F_1 from various ATPases have been ongoing for almost two decades, and it was only recently that a 2.8-Å crystal structure of the F_1 portion of F_1F_o-ATPase from bovine heart mitochondria was determined.

F_1-ATPase is a 371-kDa complex of five different subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$. The α and β subunits are arranged in an alternating fashion around a pseudosymmetric three-fold axis to make a structure shaped like a slightly squashed sphere (Fig. 17.1.35A). The smaller δ and ε subunits are not visible in the crystal structure, and only a portion of the γ subunit structure is observed in the center of the complex. The α and β subunits are structurally very homologous, with marginal overall sequence identity (20%) between them. They each consist of three domains: an N-terminal six-stranded antiparallel β barrel, a central, nine-stranded, mostly parallel β sheet surrounded on both faces by nine helices, and a C-terminal six- to seven-helix bundle (Fig. 17.1.35B). The central domain of both α and β subunits binds mononucleotide, although only the β subunit is catalytic. Both subunits have a P loop with a conserved mononucleotide-binding sequence motif at the C-terminal end of the sheet (see discussion of Nucleotide-Binding Proteins). In the center of the F_1 complex, a curving 90-Å-long α helix from the γ subunit is situated approximately parallel to the pseudo-three-fold axis. A shorter 45-residue α helix, also from the γ subunit, forms a left-handed antiparallel coiled coil with the lower half of the long helix (Fig. 17.1.35B). The only other piece of the γ subunit crystallographically visible is an 18-residue section containing a three-turn α helix located to one side of the central helices. The coiled-coil portion of the γ subunit extends 30 Å from the bottom of the $\alpha_3\beta_3$ complex.

Unlike the α subunits that each bind one molecule of the nonhydrolyzable ATP analog adenosine-5′- (β, γ-imido) triphosphate (ADPNP) symmetrically, the three β subunits are asymmetric with regard to their nucleotide binding. One β subunit binds ADPNP, another binds ADP, and the third is empty. An analysis of the asymmetry of the F_1-ATPase crystal structure indicates that the orientation of the $\alpha_3\beta_3$ subunits relative to the asymmetric γ subunit affects the conformation of the nucleotide-binding sites in the catalytic subunits, rendering each of them tight, loose, or open. It is proposed that rotation of the $\alpha_3\beta_3$ complex relative to the rest of the ATPase would cause the cycling of catalytic sites through different binding states, and hence ATP synthesis may require proton transport–linked rotation of the $\alpha_3\beta_3$ complex.

Proteasome

The structure of a 20S proteasome from *Archaeon* has been determined recently by X-ray crystallography. It forms an overall barrel-shaped structure with four rings stacked on top of each other. The two outer rings consist of seven α subunits each, and the two inner rings consist of seven β subunits each. This $\alpha_7\beta_7\beta_7\alpha_7$ assembly produces a cylindrical barrel with dimensions of 150 Å in height and 110 Å in diameter. The subunits α and β share the same fold, with a core β sandwich flanked by two α helices on each side. The core β sandwich is composed of two five-stranded antiparallel β sheets with strand order of 8–1–2–9–10 in one sheet and 3–4–5–6–7 in the other.

Viral-coat proteins of spherical viruses

The capsid proteins of a virus provide a protective shell around the enclosed RNA or DNA. These viral coats are made from an assembly of identical or nearly identical small protein subunits. The protein capsids of all known spherical viruses are built using icosahedral symmetry. In its simplest form an icosahedron is a 20-sided polygon with two-fold, three-fold, and five-fold symmetry made from an arrangement of identical equilateral triangles (Fig. 17.1.36A). Because constructing a

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Table 17.1.19 Integral Membrane Proteins

Protein	PDB entry	Reference
Bacteriorhodopsin	1pcr	Henderson et al., 1990. *JMB* 213:899-929
Rhodopseudomonas viridis photosynthetic reaction center		Deisenhofer and Michel, 1989. *Science* 245:1463-1468
Bacterial porin	2por	Weiss et al., 1991. *Science* 254:1627-1630
Light-harvesting complex from photosynthetic bacteria		McDermott et al., 1995. *Nature* 374:517-523
E. coli porins		Cowan et al., 1992. *Nature* 358:727-733
Paracoccus denitrificans cytochrome *c* oxidase		Iwata et al., 1995. Nature 376:660-669
Cytochrome *c* oxidase		Tsukihara et al., 1996. Science 272:1136-1141

protein subunit with equilateral three-fold symmetry is difficult, the simplest known viruses contain three asymmetric subunits per triangle or 60 subunits per virus. Larger and more complex viral coats can be made by further subdividing these asymmetric units and using more protein subunits, all the while maintaining icosahedral symmetry. Viruses can be classified by their triangulation (T) number which indicates the number of capsid subunits in multiples of 60. For example a T = 1 virus has 60 subunits and a T = 3 virus has 3 × 60 = 180 subunits.

The three-dimensional structures of numerous capsid proteins for spherical viruses that infect bacteria, plants, insects, and animals have revealed a common fold for most of these structures—i.e., an eight-stranded jelly roll β sandwich (see discussion of Elements of Protein Structure). Strands 1–8–3–6 form one sheet and strands 2–7–4–5 form the other sheet of the β sandwich (Fig. 17.1.36B). Despite the lack of sequence homology between various classes of viral-coat proteins, the jelly roll β sandwich topology is well conserved in at least 22 known capsid structures archived in the PDB (Murzin et al., 1995). It forms a wedge-shaped β sandwich, generally with short loops at one end and longer loops at the other end. A striking exception to this fold is observed in the coat protein of bacteriophage MS2, which is a T = 3 virus (see discussion of RNA-Binding Structural Motifs and Domains).

Integral Membrane Proteins

Although the number of three-dimensional structures of water-soluble proteins that have been determined is growing exponentially every year, the number of membrane-protein structures available remains scarce. To date, there are only about twenty or so such structures available in less than ten different folds, as compared to more than a thousand water-soluble protein structures with >200 different folds. Integral membrane proteins are often difficult to crystallize, and their crystallizations are particularly sensitive to the choice of detergents. Among the few classes of membrane protein structures available today are the structure of bacteriorhodopsin from *Halobacterium halobium*, the photosynthetic reaction center from *Rhodobacter viridis*, several bacterial porins and maltoporin, light-harvesting complex from purple bacterium, cytochrome *c* oxidase from both bacterium and bovine heart, and a *Staphylococcus* α-hemolysin (see Table 17.1.19 for specific references). Most of them present rather simple folds in the transmembrane domains, either all α-helical or all β-sheet, although the actural number of transmembrane helices and strands varies in each case.

The members of the family of all-α-helical integral membrane proteins include bacteriorhodopsin (Fig. 17.1.37A), photosynthetic reaction center (Fig. 17.1.37B), light harvest complex, and cytochrome *c* oxidase. The number of membrane-spanning helices differs from three helices in the light harvest complex (LHC-II), seven in bacteriorhodopsin, and eleven in the photosynthetic reaction center, to 22 spanning helices in the cytochrome *c* oxidases.

There are only two examples of all-β-sheet folds in membrane proteins that have been determined as of today. These are the structures of porins/maltoporins and the structure of an α-hemolysin (Fig. 17.1.37C). In both cases, the transmembrane β strands form antiparallel closed β barrels. The number of strands in each barrel varies, with 16 in the structure of porin and 14 in α-hemolysin.

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Figure 17.1.1 *(at right)* (**A**) Drawing of an L-polypeptide chain using a ball-and-stick model to illustrate torsion angles ϕ and ψ for residue *i*. Torsion angle ϕ defines the angle between the planes specified by atoms $C_{i-1} - N_i - C_i^{\alpha}$ and $N_i - C_i^{\alpha} - C_i$, respectively. Torsion angle ψ defines the angle between the plane specified by atoms $N_i - C_i^{\alpha} - C_i$ and $C_i^{\alpha} - C_i - N_{i+1}$ respectively. (**B**) Ball-and-stick (left) and ribbon (right) representations of an α helix. (**C**) Ball-and-stick and ribbon representations of β sheet in both antiparallel and parallel orientations. (**D**) Illustration of the characteristic right-handed twist of a β sheet as observed in flavodoxin (PDB entry 1flv). (**E**) Type I and II tight turns.

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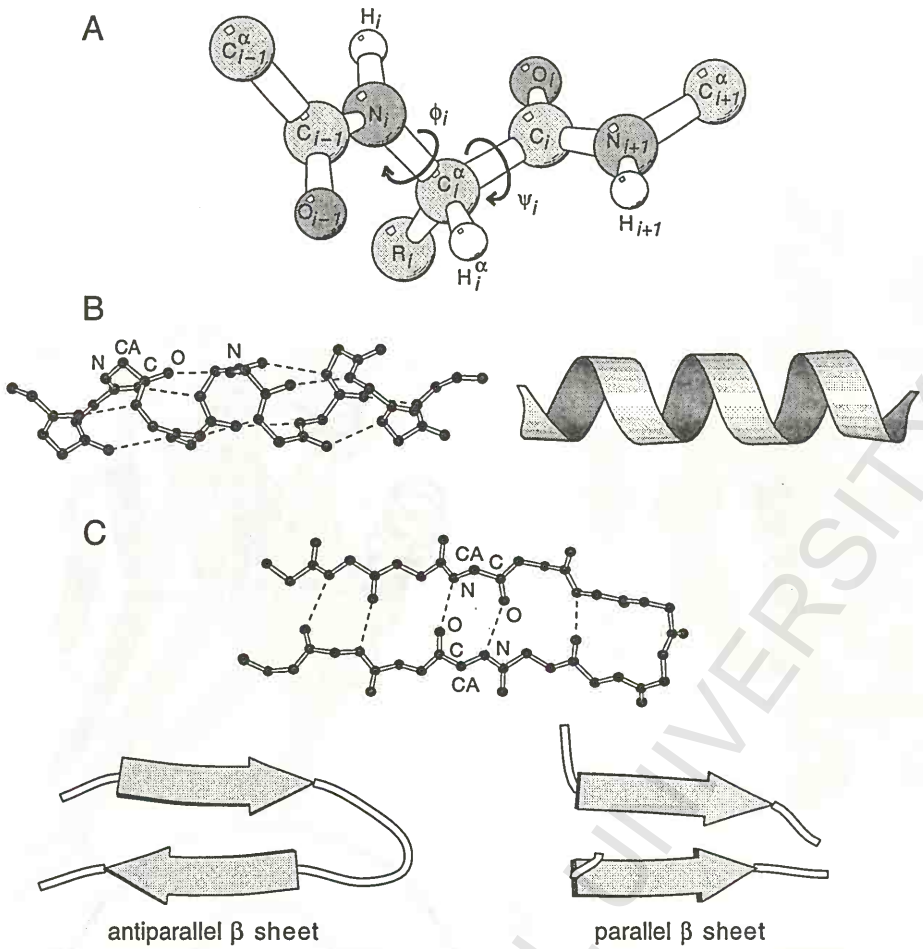

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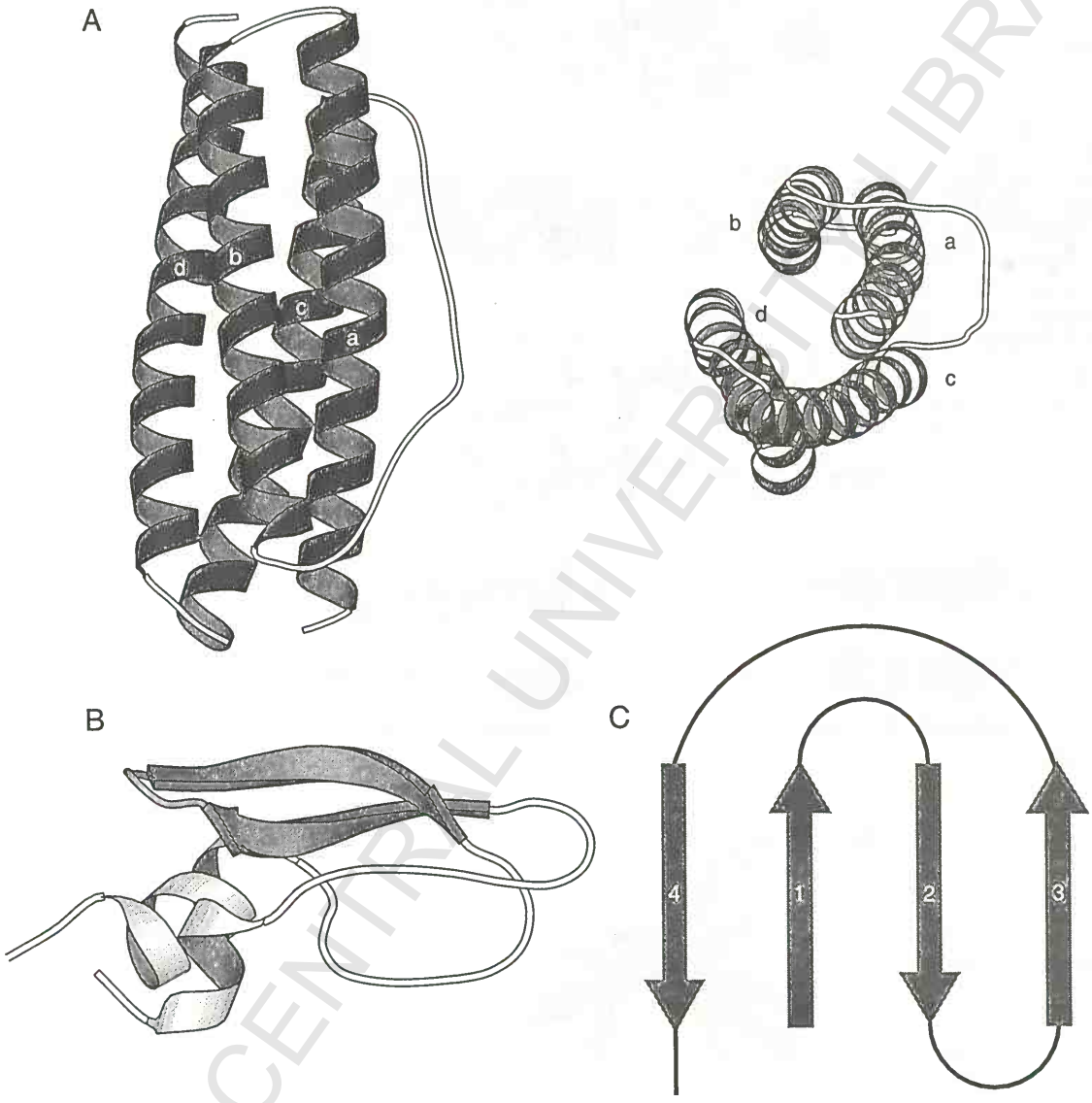

Figure 17.1.2 *(above and at right)* Examples of commonly observed secondary structure assemblies. (**A**) Two orthogonal views of a four-helix bundle (PDB entry 1bcf). (**B**) A β hairpin structure (PDB entry 1bpi). (**C**) Topology diagram of a β sheet with Greek key topology. (**D**) A jelly-roll motif (PDB entry 1pgs). (**E**) A β sandwich (PDB entry 4gcr). (**F**) A β barrel (PDB entry 2por). (**G**) An α/β barrel (PDB entry 1btm).

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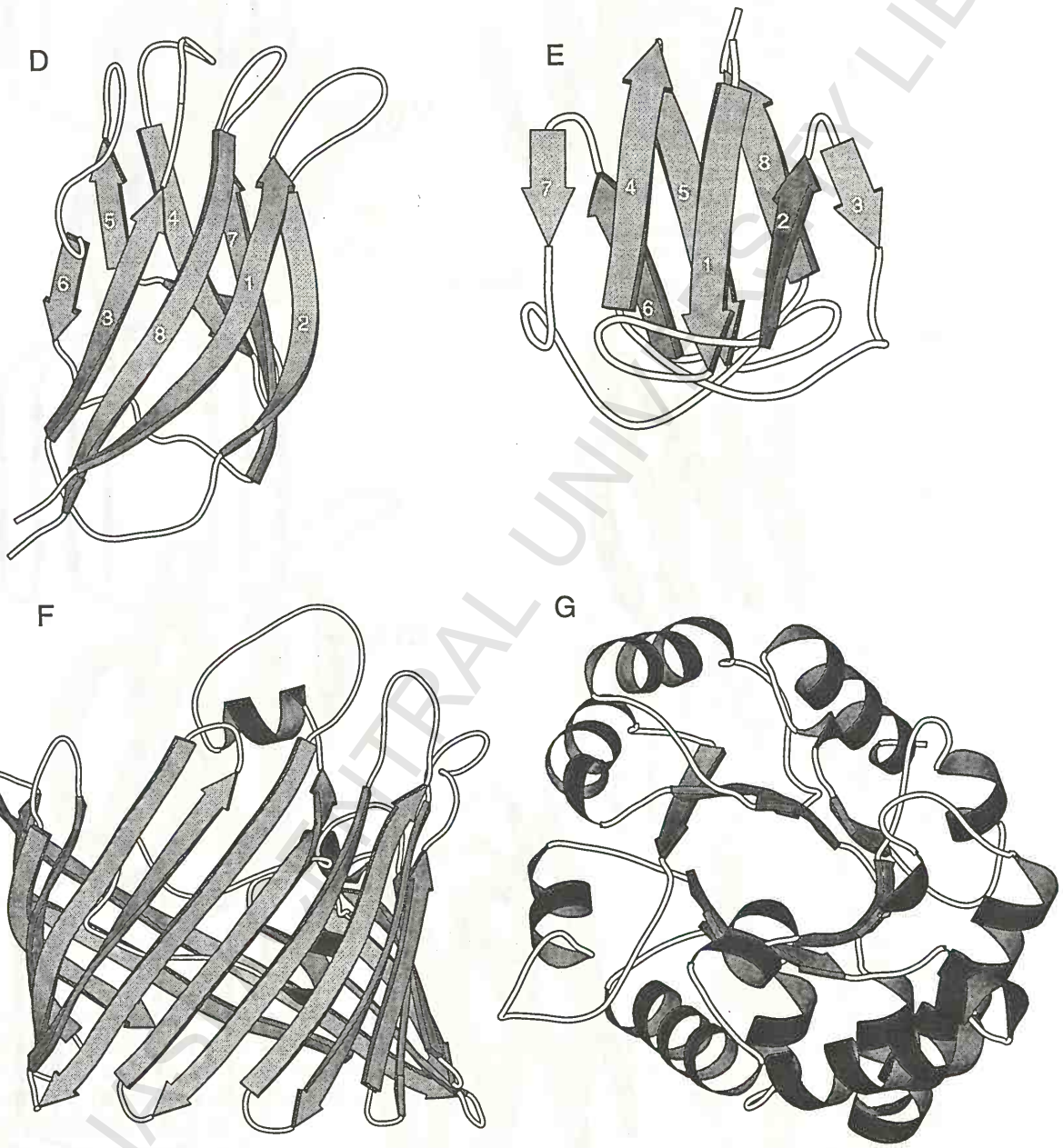

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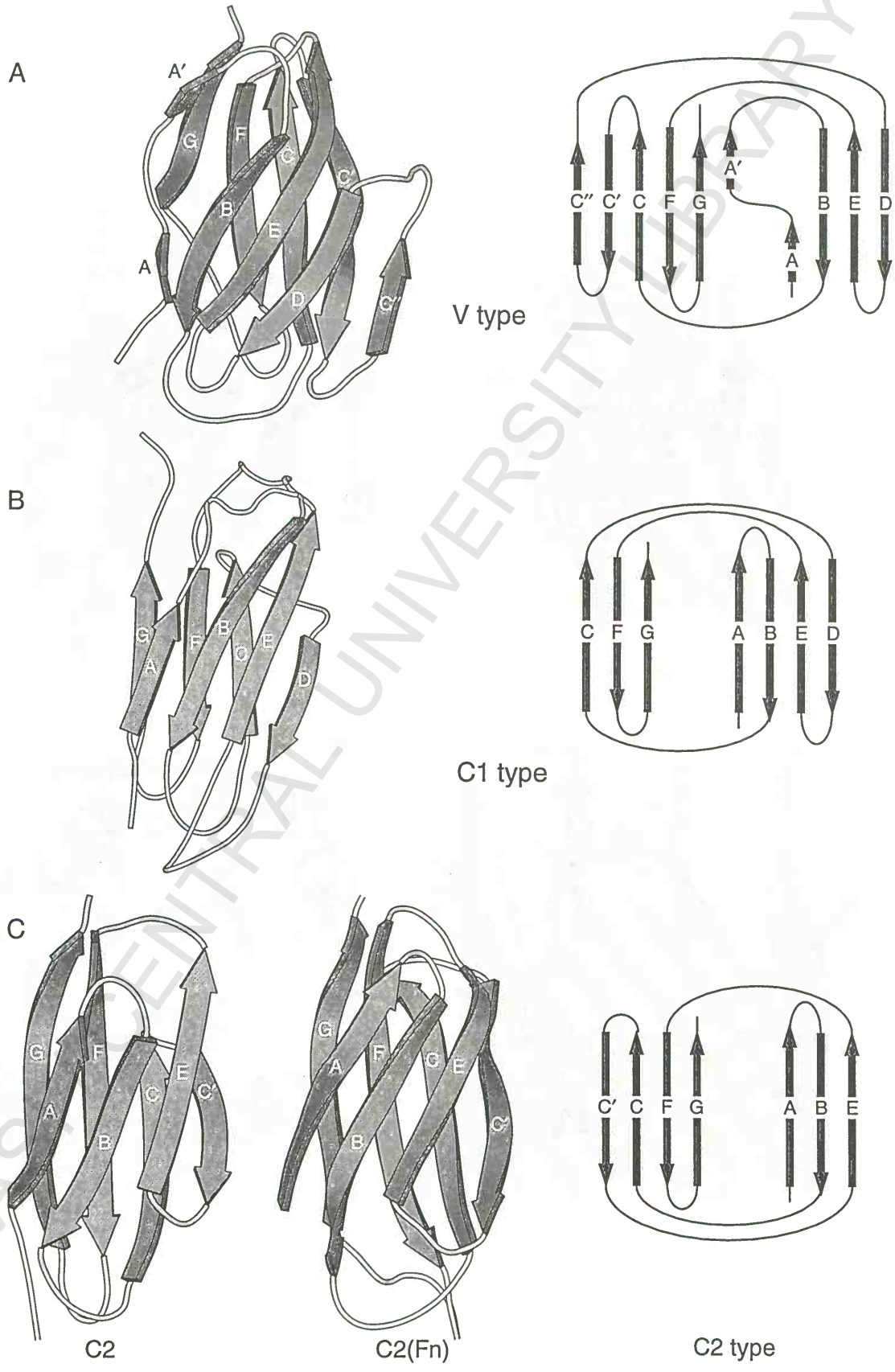

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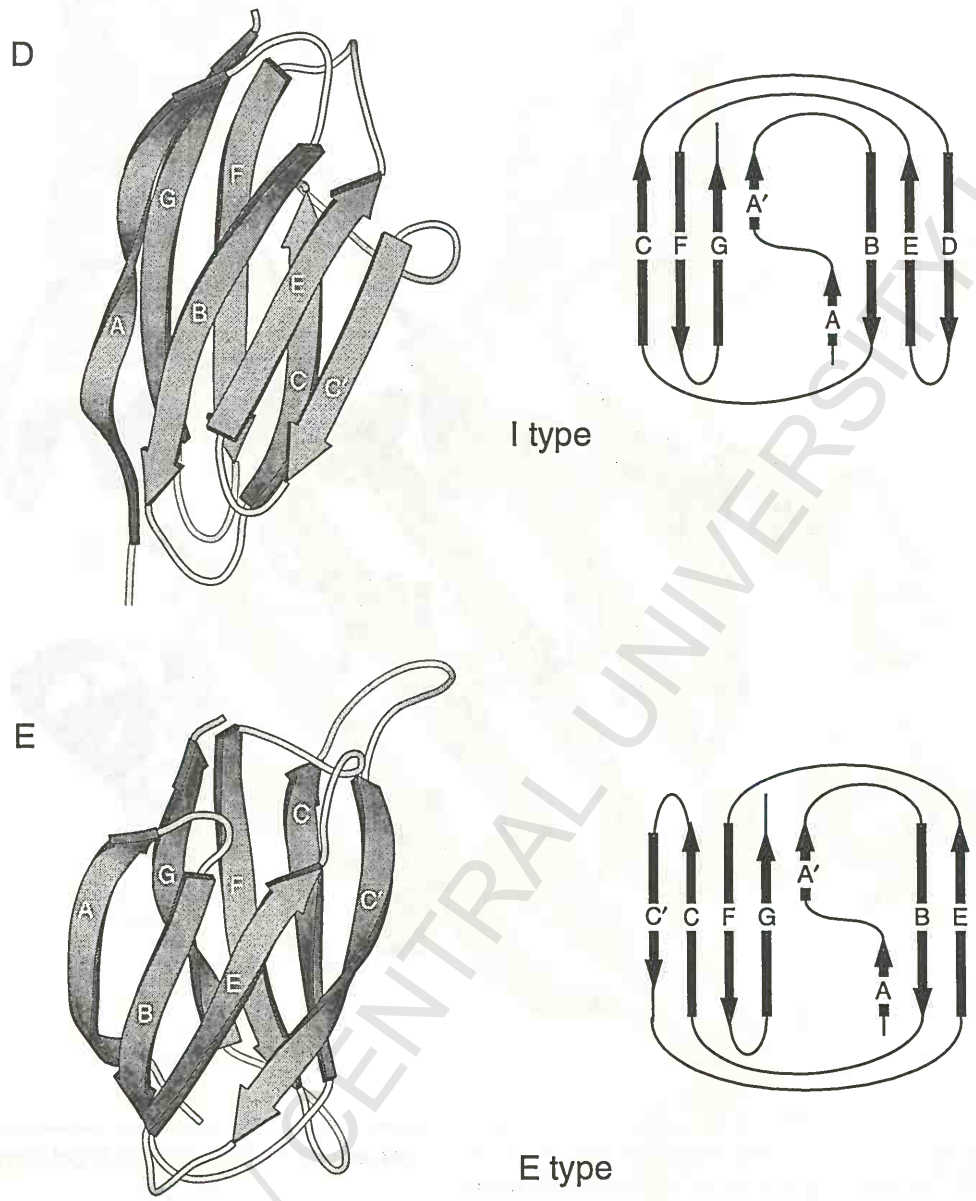

Figure 17.1.3 *(above and at left)* Tertiary and secondary structures of immunoglobulin fold. The coordinates used for the ribbon diagrams are taken from the PDB entries indicated. (**A**) V (PDB entry 3hfl), (**B**) C1 (PDB entry 3hfl), (**C**) C2/C2(Fn) (PDB entry 1hnf), (**D**) I type (PDB entry 1fna), and (**E**) E type (PDB entry 1flk).

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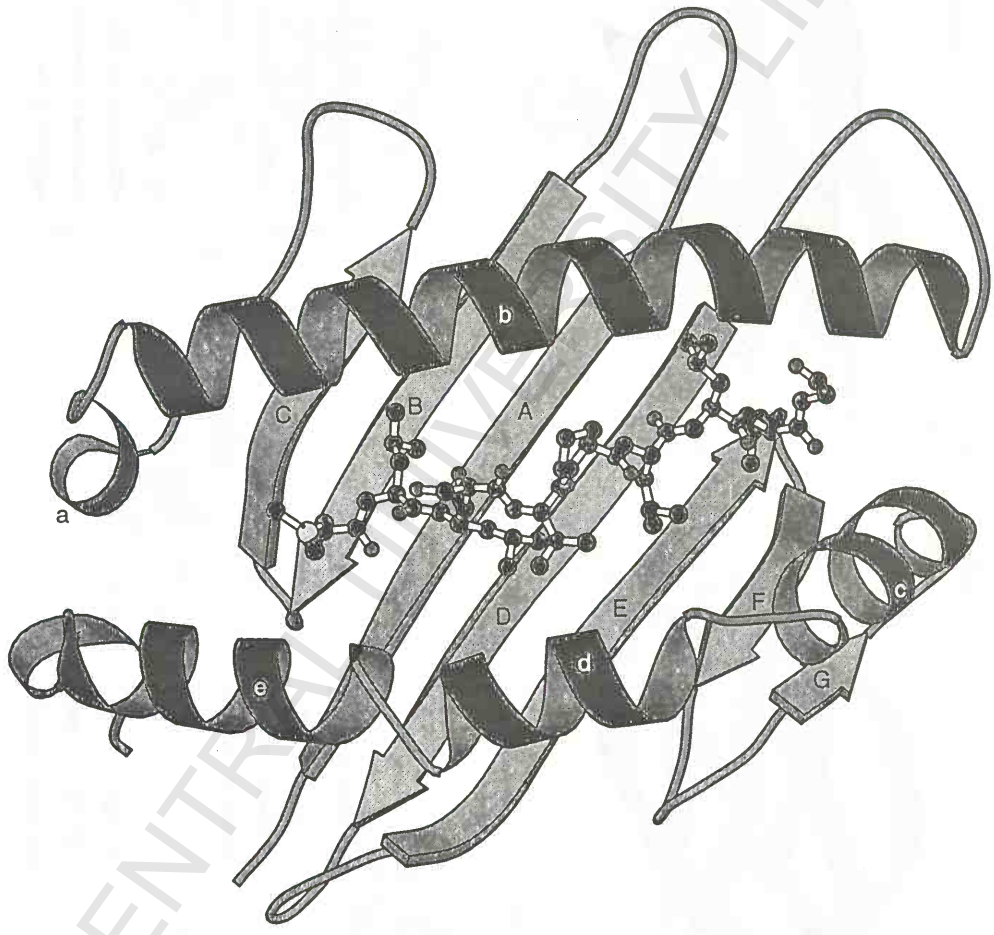

Figure 17.1.4 Structure of a class I MHC HLA-A2 complex with an antigenic peptide (PDB entry 2clr). The peptide is shown as a ball-and-stick model.

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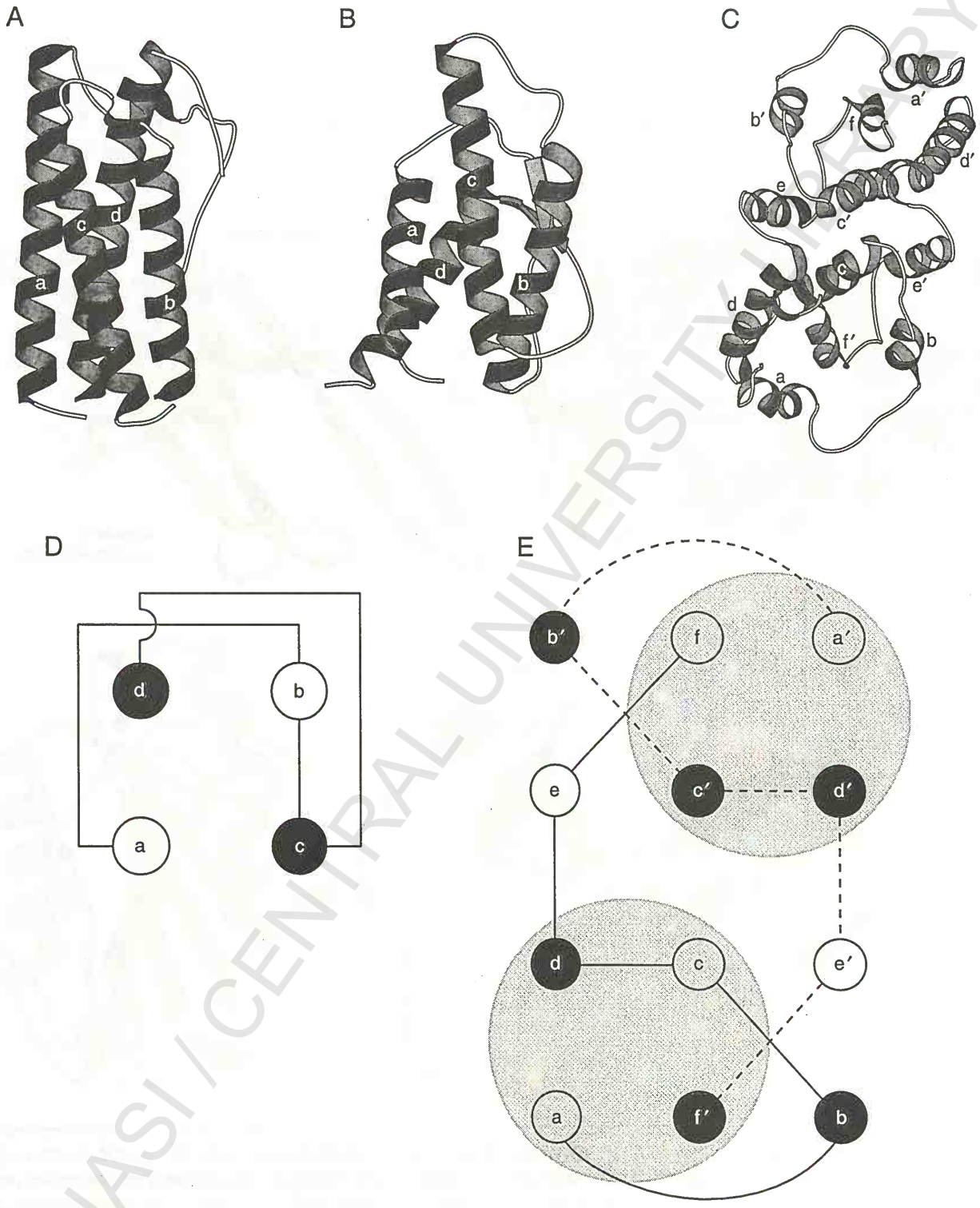

Figure 17.1.5 The structure of (**A**) a long-chain helical cytokine, GCSF (PDB entry 1rhg), (**B**) a short-chain helical cytokine, IL-4 (PDB entry 1rcb), and (**C**) interferon γ (PDB entry 1rfb). (**D**) Connectivity between helices in four-helix bundle cytokines. The "up helices" are drawn in white and the "down helices" are in black. (**E**) Connectivity between helices of IFN-γ. The gray-shaded regions correspond to the four-helix bundles.

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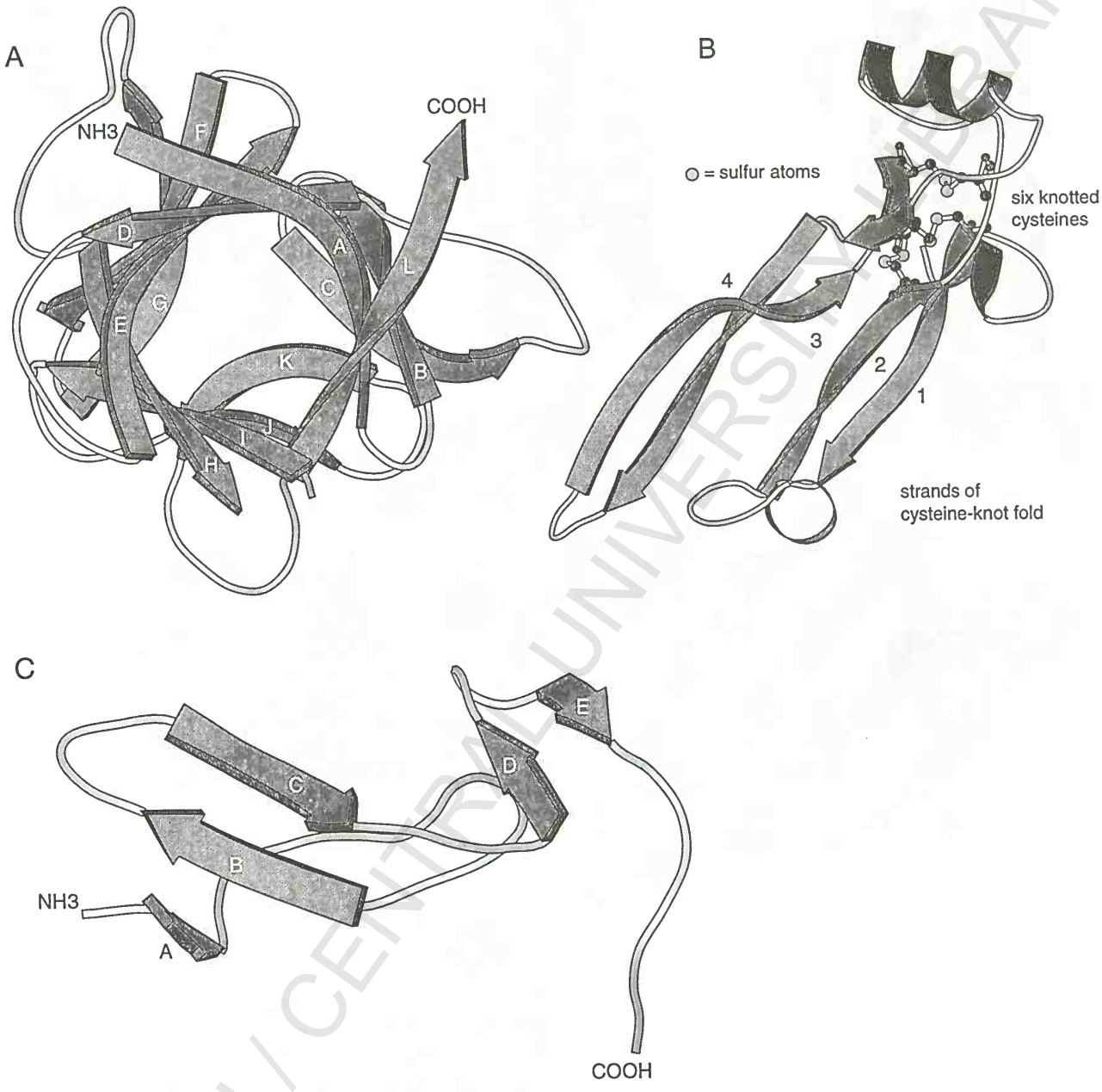

Figure 17.1.6 (**A**) Structure of a human IL-1β, a member of the β-trefoil fold (PDB entry 1hib). (**B**) Crystal structure of a human transforming growth factor β2 (PDB entry 2tgi). The six knotted cysteines are shown in ball-and-stick model (with the sulfur atoms as gray balls). (**C**) The structure of an EGF domain (PDB entry 1epj).

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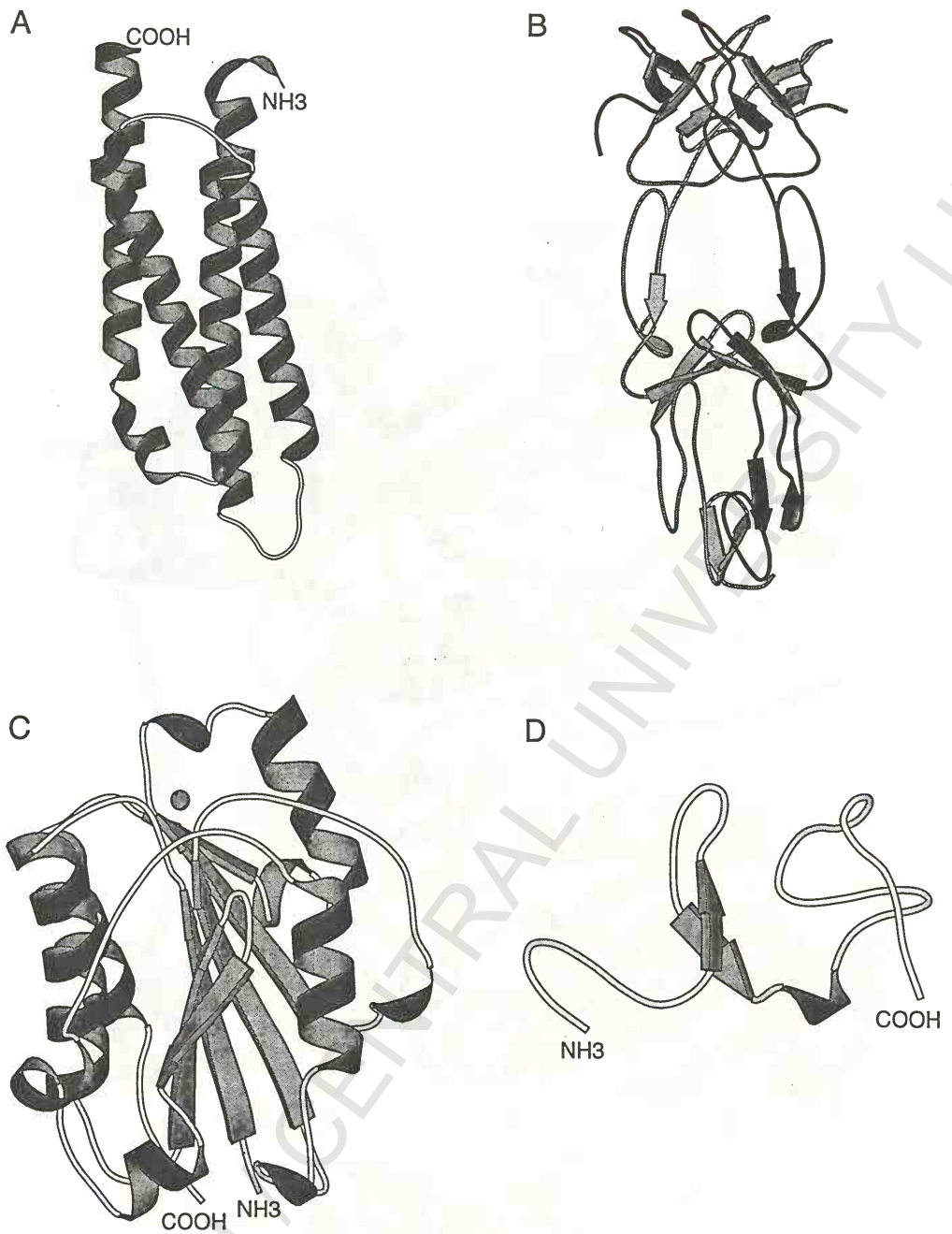

Figure 17.1.7 Structures of (**A**) aspartate receptor (PDB entry 1wat), (**B**) a dimer of TNF receptor with monomers differently shaded (PDB entry 1ncf), (**C**) Integrin I domain (PDB entry 1lfa), and (**D**) LDL receptor (PDB entry 1ldl).

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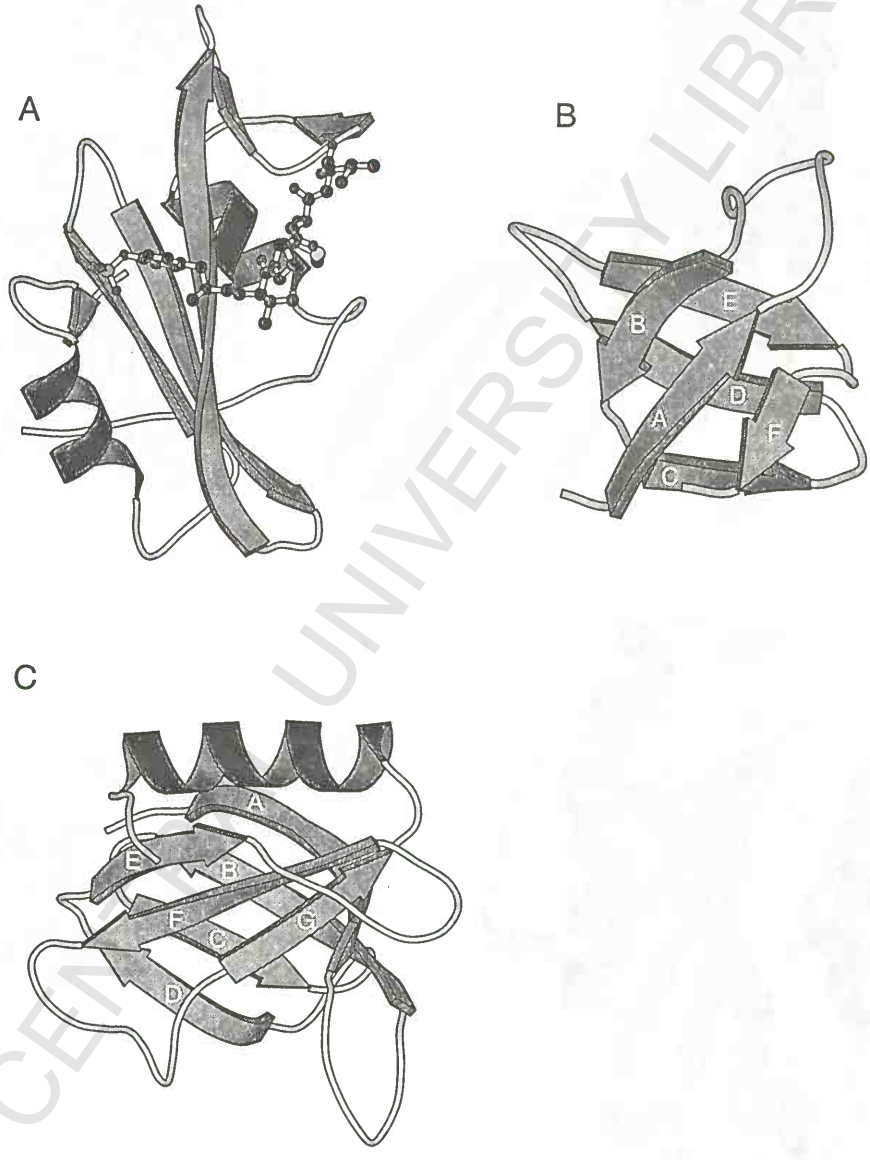

Figure 17.1.8 *(above and at right)* Structures of (**A**) SH2 domain in complex with a phosphotyrosine peptide (PDB entry 1sha), (**B**) SH3 (PDB entry 1csk), and (**C**) PH (PDB entry 1dyn) domains. (**D**) Crystal structure of a phosphoylated cyclic AMP-dependent protein kinase (cAPK) complexed with an ATP, Mn, and a peptide inhibitor. The ATP molecule, Mn, and phosphoylated serine and threonine are shown in ball-and-stick representation. The peptide inhibitor is shown as the black coil superimposed over the structure on the right (PDB entry 1atp).

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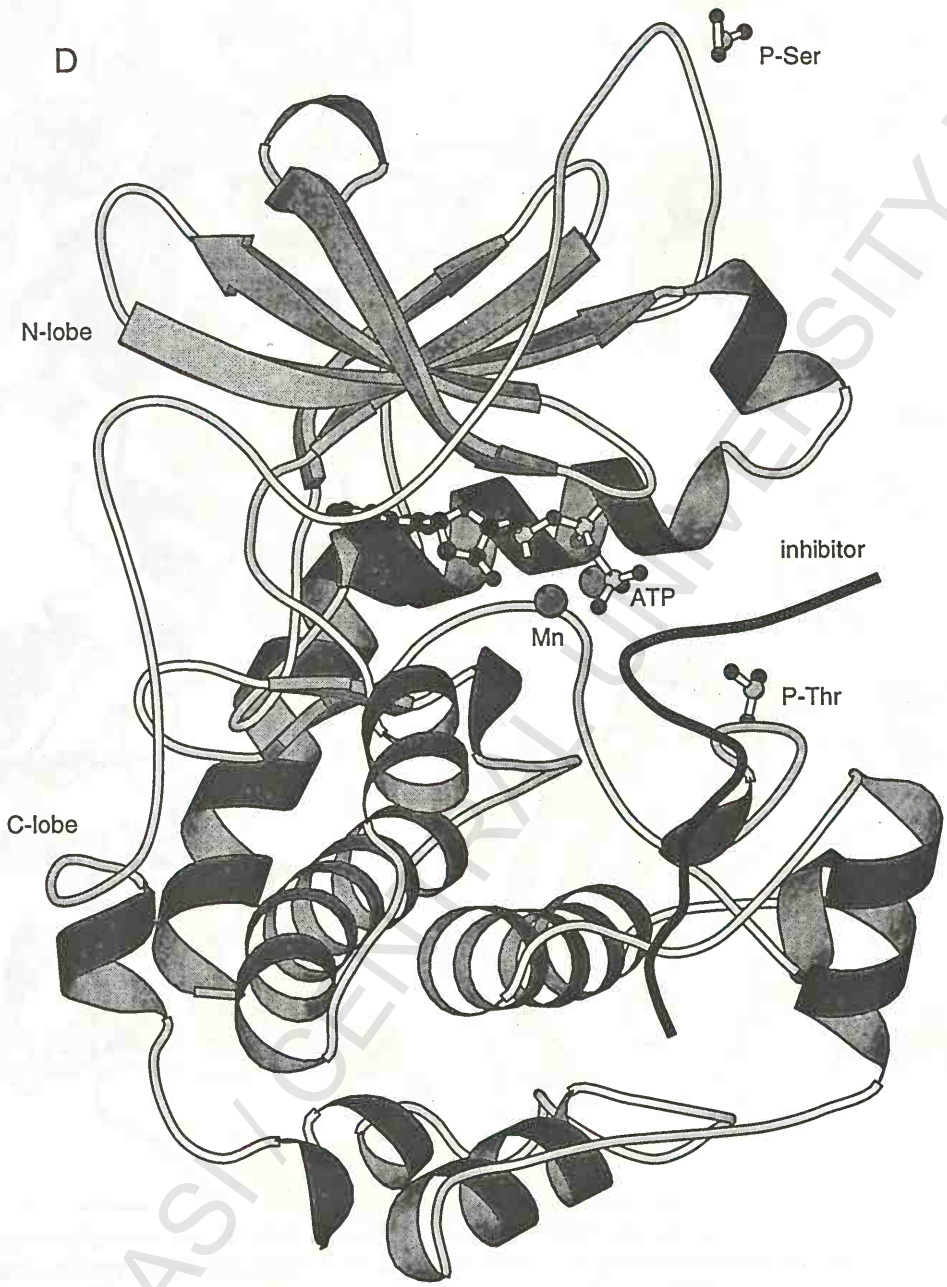

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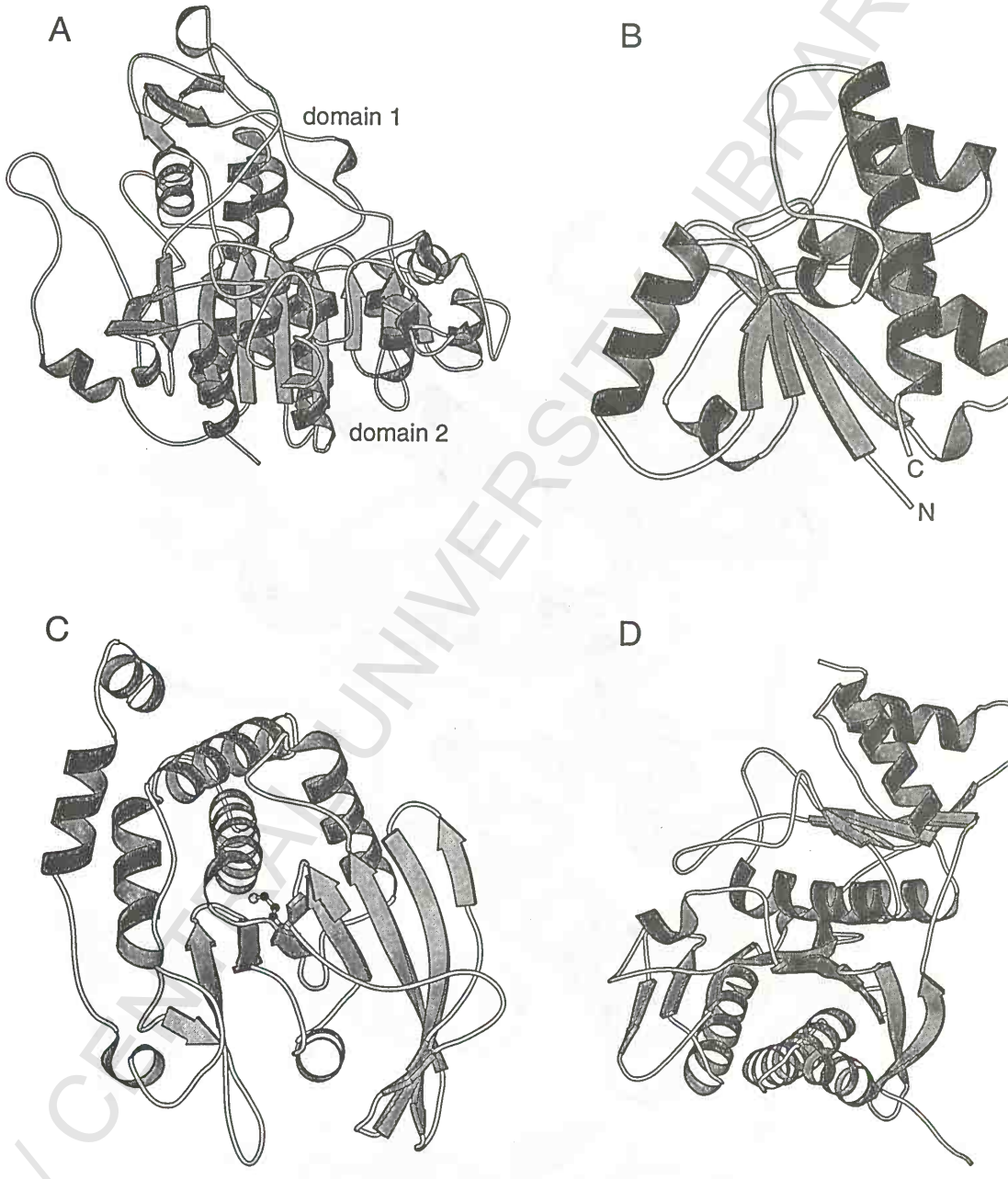

Figure 17.1.9 Structures of (**A**) alkaline phosphatase (PDB entry 1ali), (**B**) low-molecular-weight protein phosphotyrosine phosphatase (PDB entry 1phr), (**C**) high-molecular-weight protein phosphotyrosine phosphatase (PDB entry 2hnq), and (**D**) sugar phosphatase (PDB entry 5fbp).

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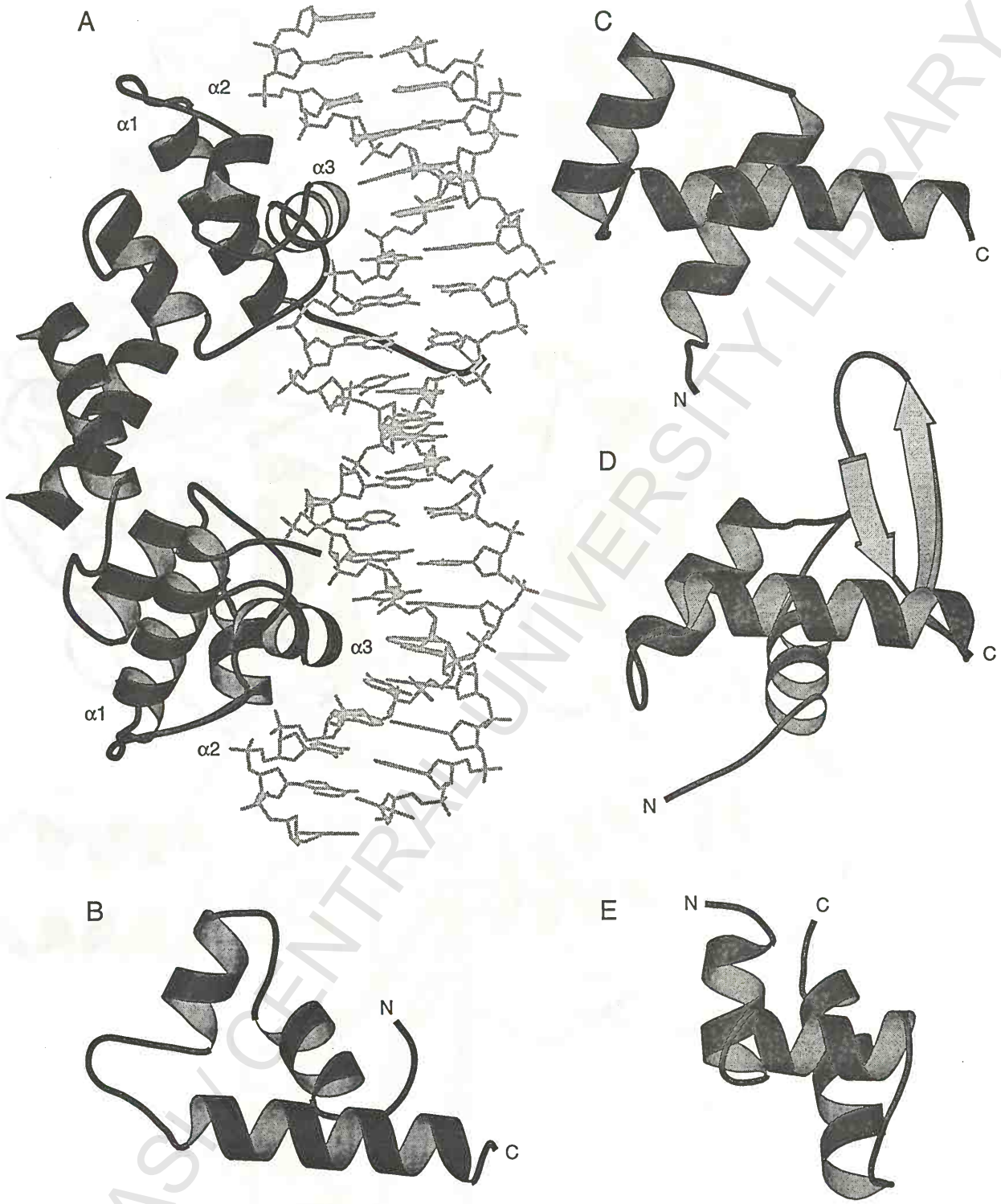

Figure 17.1.10 Variations of the helix-turn-helix (HTH) structural motif. The recognition helix is oriented horizontally in panels **B** to **E**. (**A**) Homodimer of the DNA-binding domain of λ repressor bound to DNA (PDB entry 1lmb). The three helices of the HTH motif are labeled α1, α2, and α3 in each monomer. Both recognition helices (α3) sit in the major groove. (**B**) λ repressor (prokaryotic HTH; PDB entry 1lmb). (**C**) Engrailed homeodomain (PDB entry 1hdd). (**D**) Globular domain of histone H5 (PDB entry 1hst). This example of the winged helix motif has only one wing (the β hairpin). (**E**) Purine repressor (purR; PDB entry 1pru).

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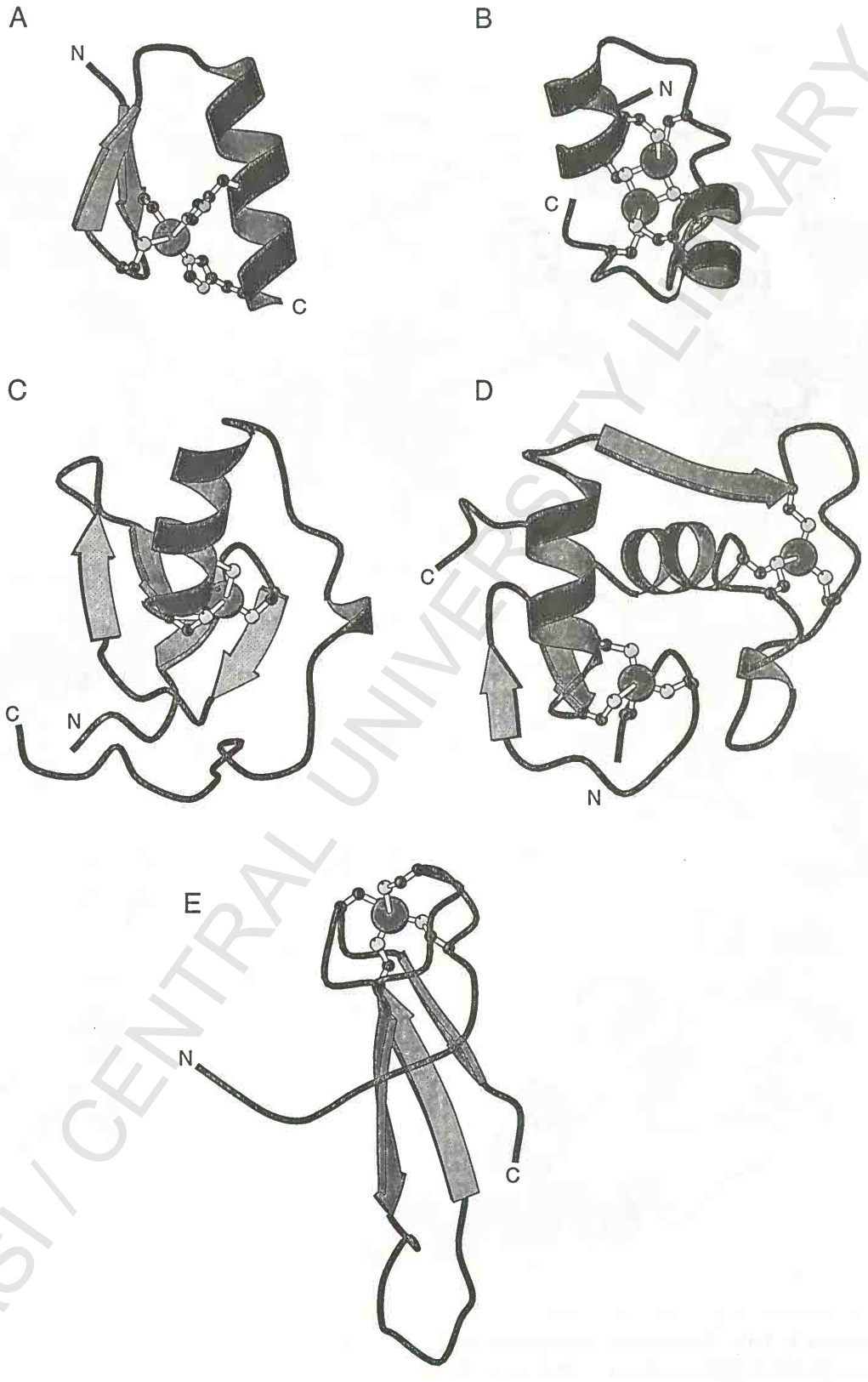

Figure 17.1.11 Zinc-binding motifs within DNA-binding domains. The zinc atom and side-chain ligands to the zinc are represented by ball-and-stick models. (**A**) Second zinc finger from ZIF268 mouse intermediate protein (PDB entry 1zaa). (**B**) Zn_2Cys_6 binuclear cluster from GAL4 (PDB entry 1d66). (**C**) GATA-1 chicken erythroid transcription factor (PDB entry 1gat). (**D**) DNA-binding module from the glucocorticoid receptor (PDB entry 1gdc). (**E**) Elongation factor TFIIS (PDB entry 1tfi).

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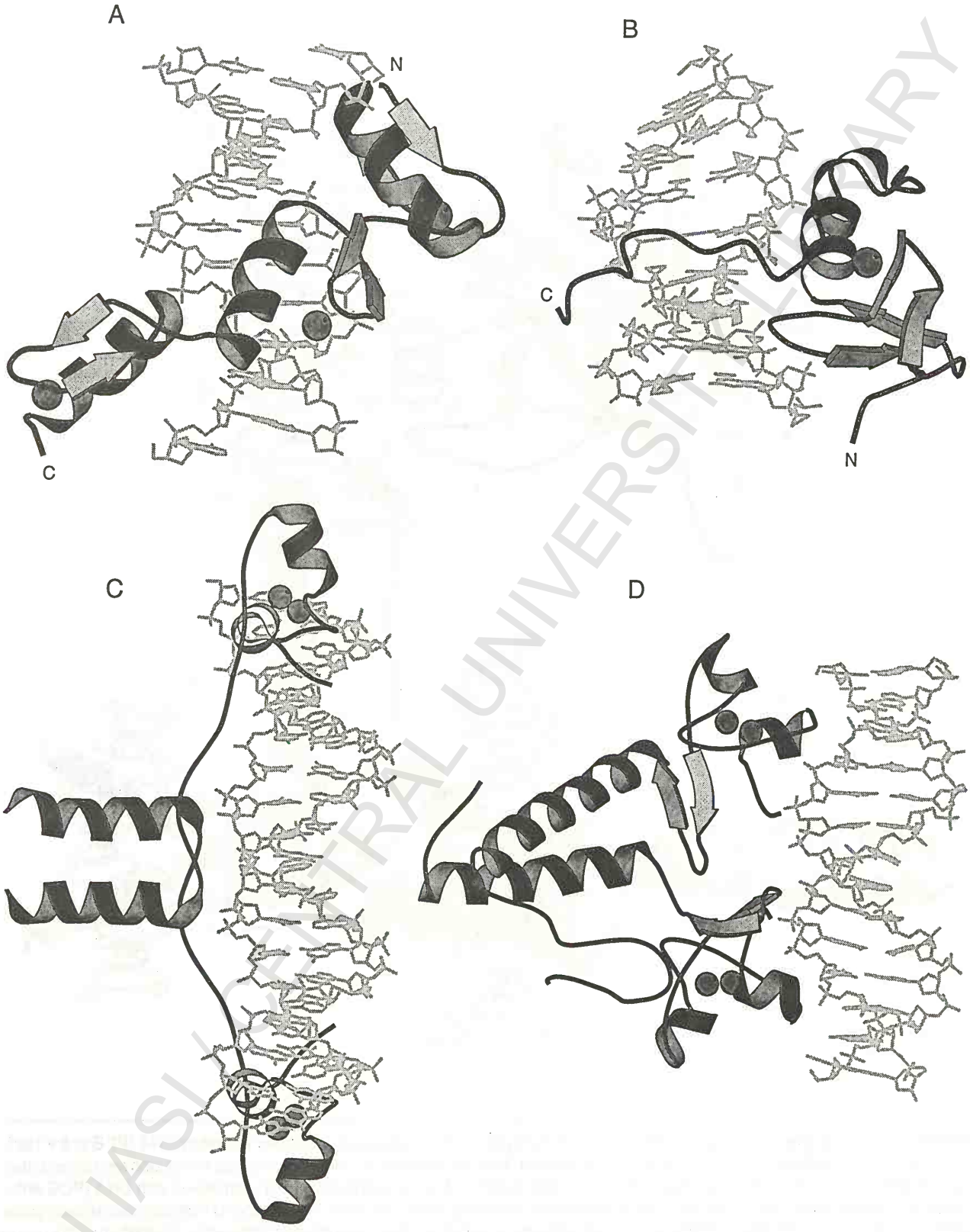

Figure 17.1.12 Binding of zinc-containing modules to DNA. The zinc atoms are represented by spheres. (**A**) The three zinc fingers of ZIF268 (PDB entry 1zaa). (**B**) GATA-1 transcription factor (PDB entry 1gat). Zn_2Cys_6 binuclear clusters bound to DNA: (**C**) Gal4 (PDB entry 1d66) and (**D**) pyrimidine pathway regulator 1 (PPR1) DNA-binding fragment (PDB entry 1pyi).

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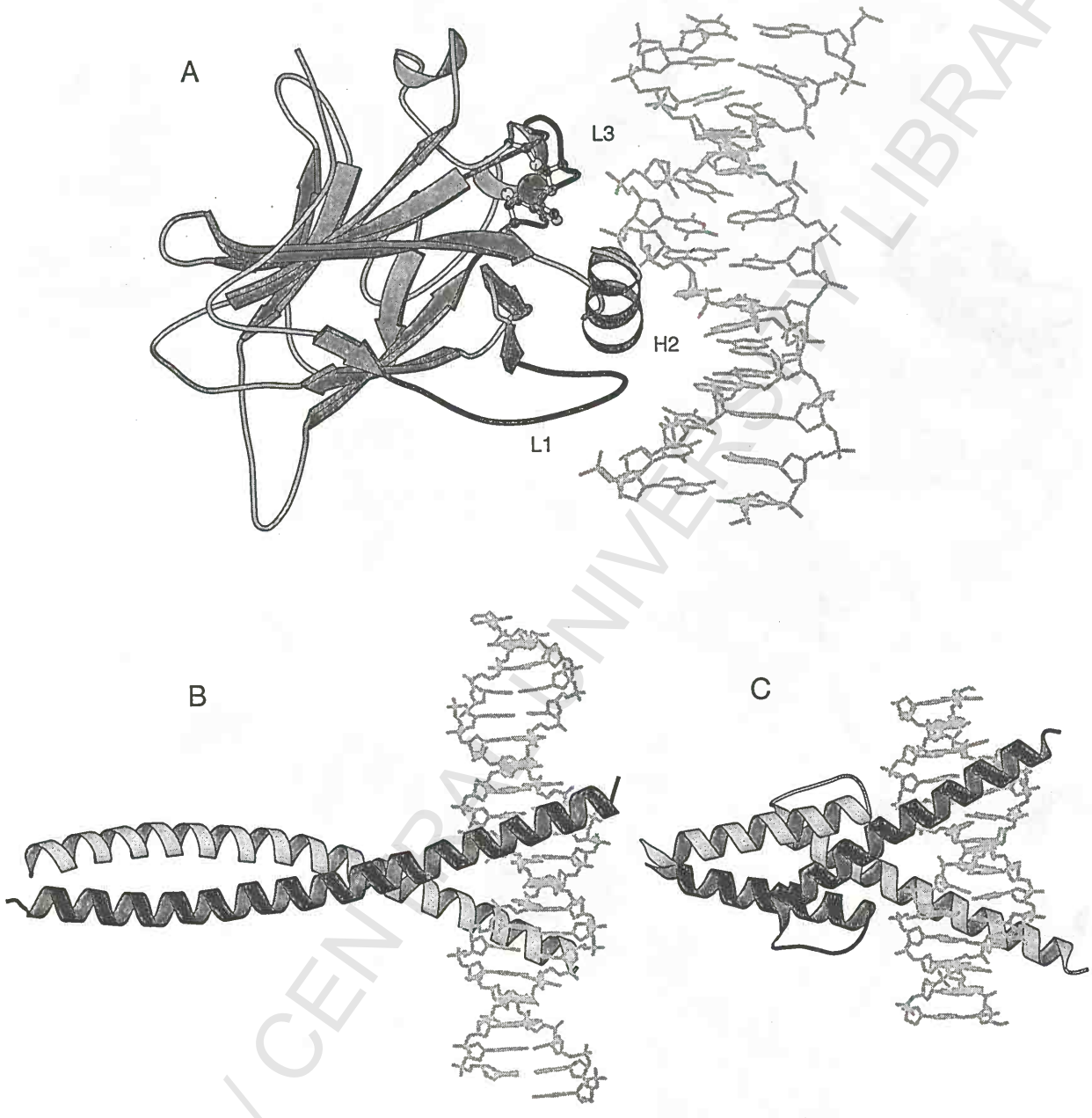

Figure 17.1.13 *(above and at right)* (**A**) The structure of p53 tumor-suppressor monomer bound to DNA (PDB entry 1tsr). Loops L1 and L3 and helix H2 are involved in DNA binding. The zinc atoms and side-chain ligands to the zinc are represented by ball-and-stick models. (**B**) Basic region leucine zipper (bZIP) c–Fos–c–Jun heterodimer complexed with DNA (PDB entry 1fos). Monomers within the dimer are shaded differently. (**C**) Basic helix-loop-helix (bHLH) MyoD homodimeric transcription activator complexed with DNA (PDB entry 1mdy). Monomers within the dimer are shaded differently. (**D**) High-mobility group (HMG) fragment B from rat (PDB entry 1hme).

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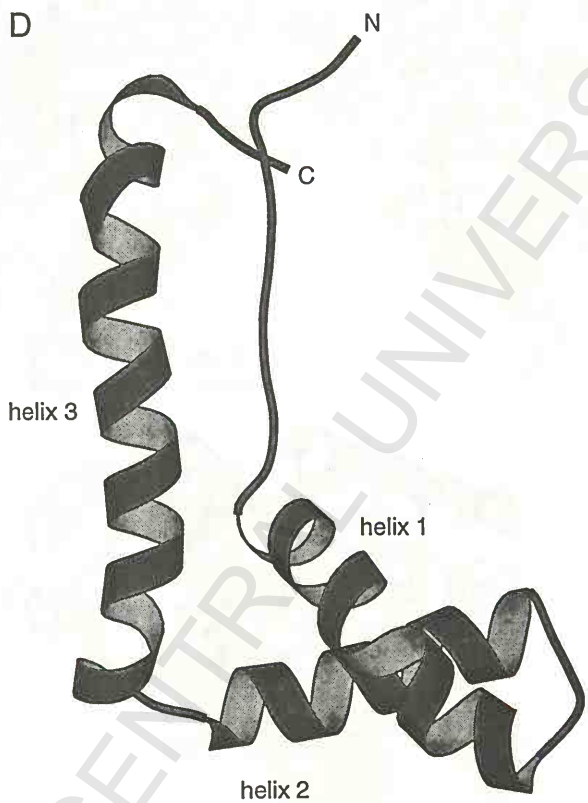

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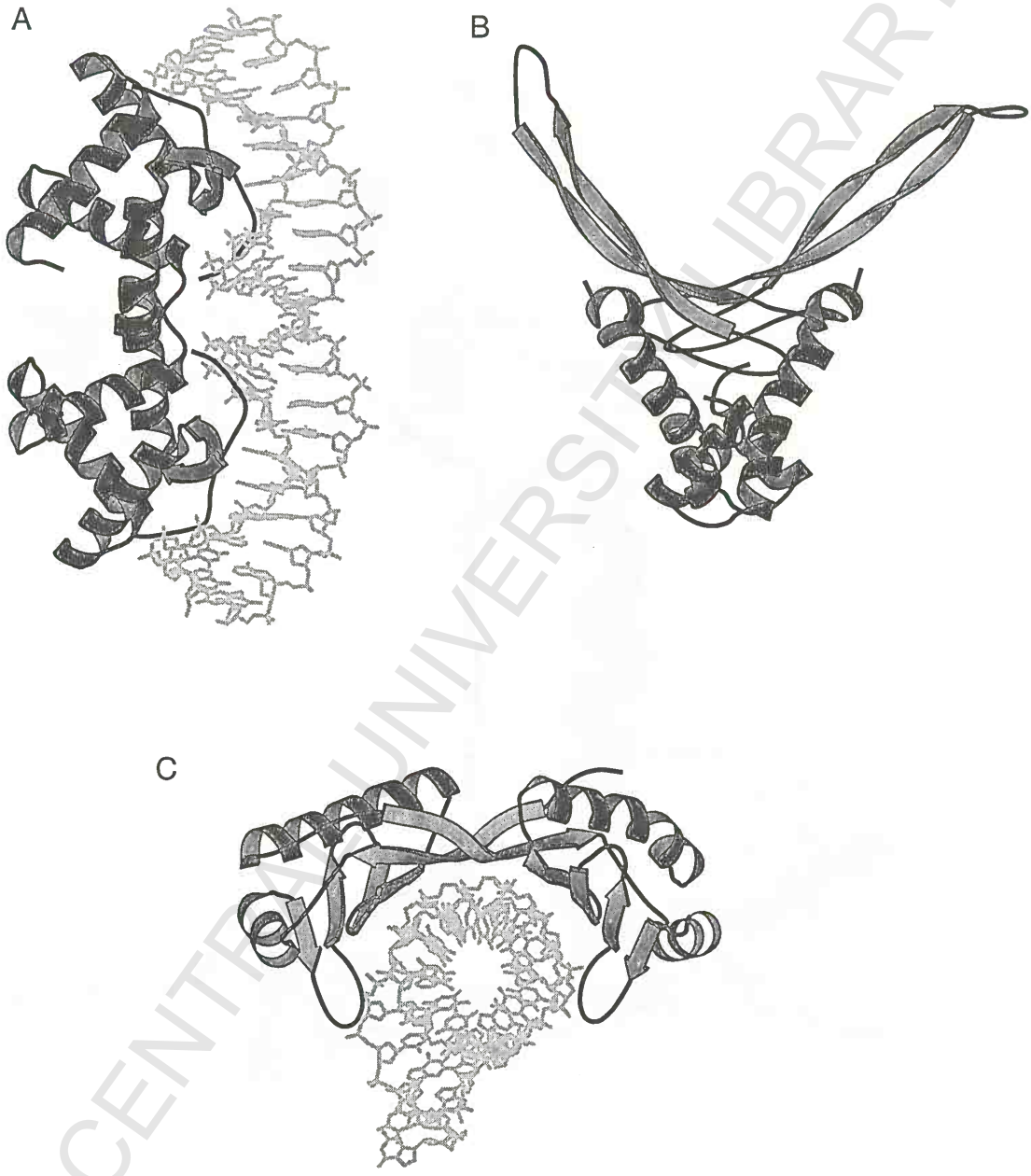

Figure 17.1.14 *(above and at right)* (**A**) Arc repressor tetramer complexed with DNA (PDB entry 1par). (**B**) Histone-like HU protein (PDB entry 1hue). (**C**) TATA-box binding protein (TBP) complexed with DNA (PDB entry 1ytb). (**D**) Structure of the p50/p50 homodimer complexed with DNA (PDB entry 1svc). The two insertion regions may play a role in binding other transcription factors. (**E**) Structure of a heterodimer of dTAF42 and dTAF62 (PDB entry 1taf). Each monomer contains the histone fold.

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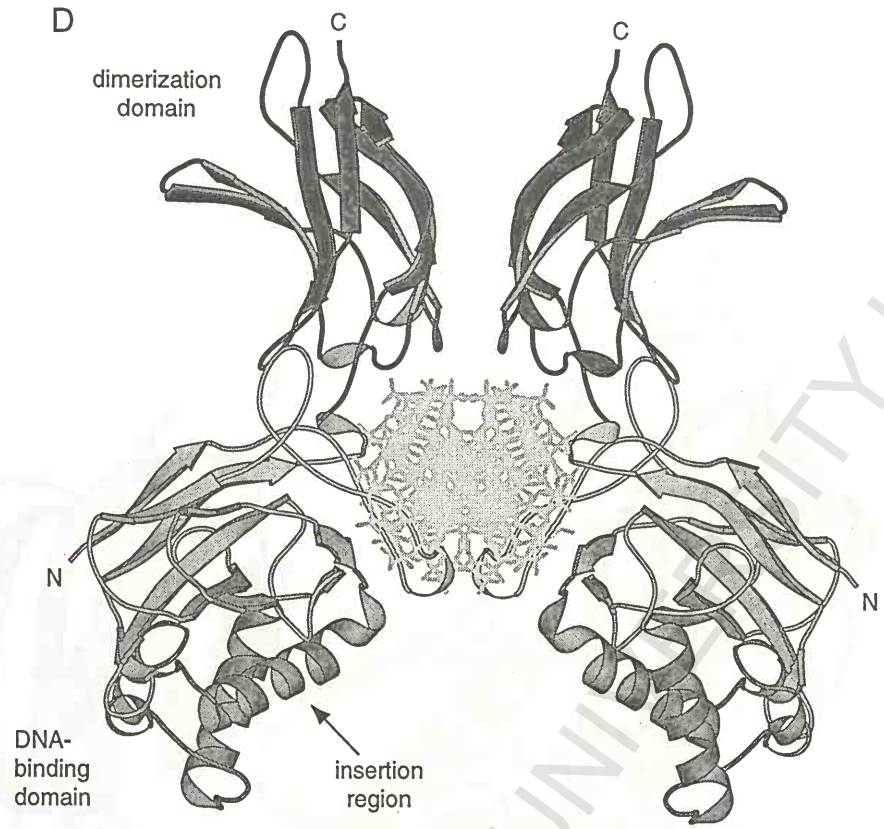

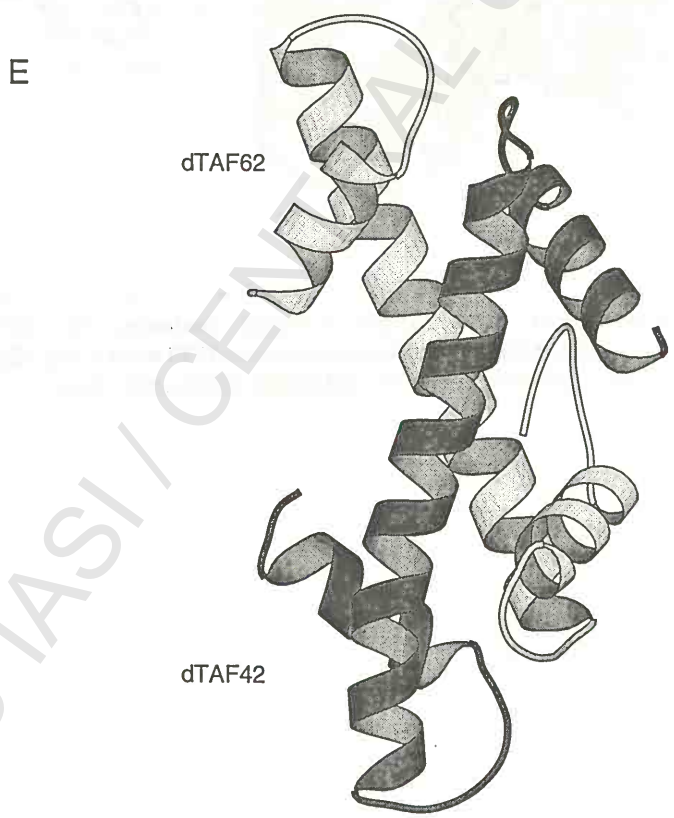

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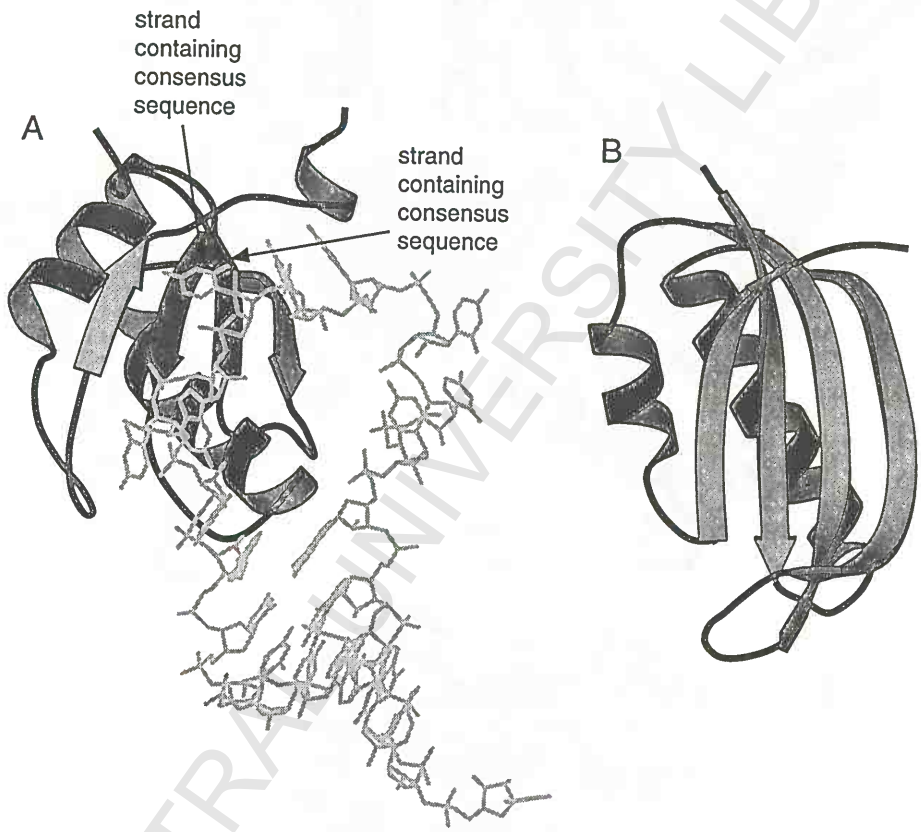

Figure 17.1.15 *(above and at right)* RNP domains. β strands containing the RNP1 or RNP2 consensus sequences are labeled. (**A**) U1A splicosomal protein (PDB entry 1urn). (**B**) Ribosomal protein S6 (PDB entry 1ris). (**C**) Bacterophage T4 regA protein (PDB entry 1reg).

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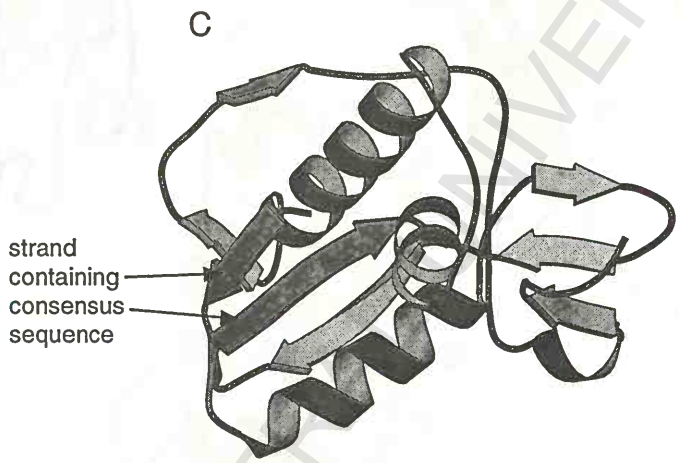

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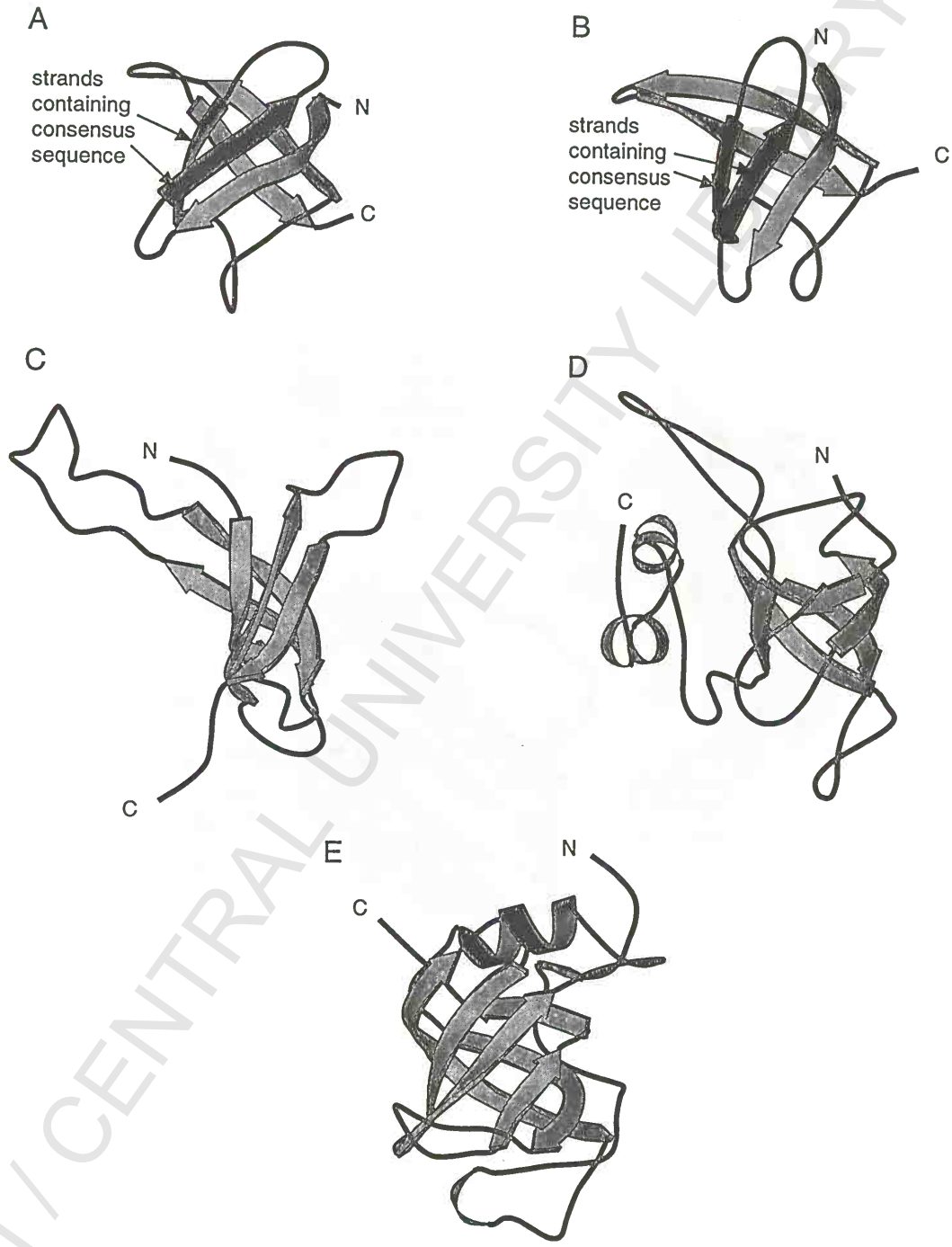

Figure 17.1.16 (**A**) Cold-shock protein A (PDB entry 1mjc). β strands containing RNP1 or RNP2 consensus sequences are labeled. (**B**) Cold-shock protein B (PDB entry 1csp). β strands containing RNP1 or RNP2 consensus sequences are labeled. (**C**) Ribosomal protein S17 (PDB entry 1rip). (**D**) Ribosomal protein L14 (PDB entry 1whi). (**E**) Anticodon-binding domain of Aspartyl-tRNA synthetase (PDB entry 1asz).

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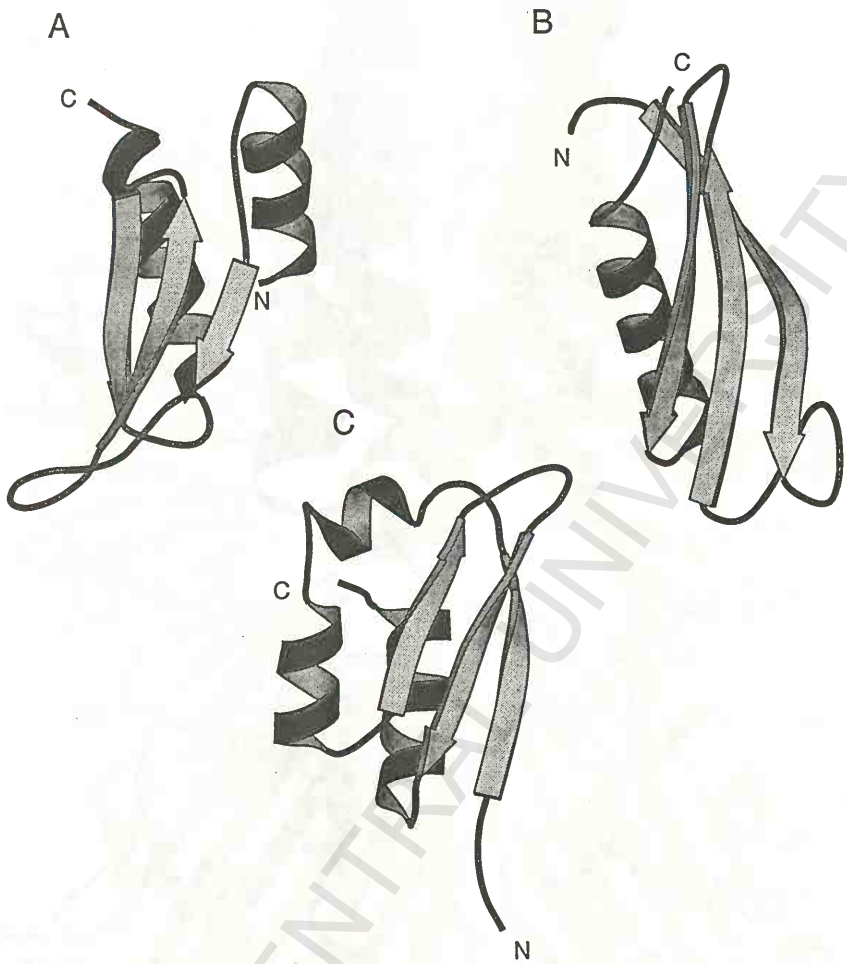

Figure 17.1.17 Double-stranded RNA-binding domain (dsRBD) and KH domain. (**A**) *Drosophila* staufen protein (PDB entry 1stu), an example of dsRBD fold. (**B**) N-terminal domain of ribosomal protein S5 (PDB entry 1pkp). (**C**) Human vigilin (PDB entry 1vih), an example of a KH domain.

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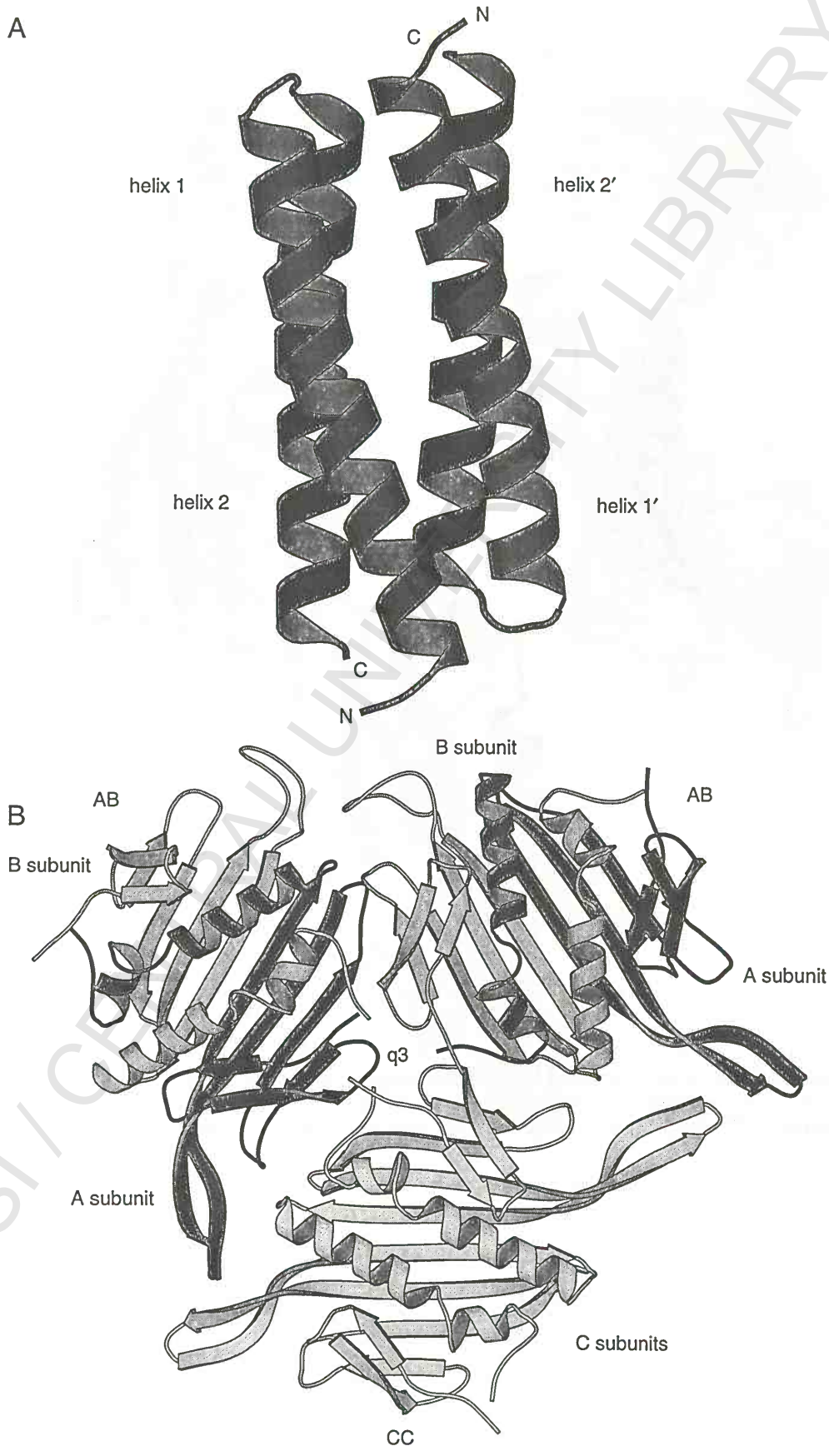

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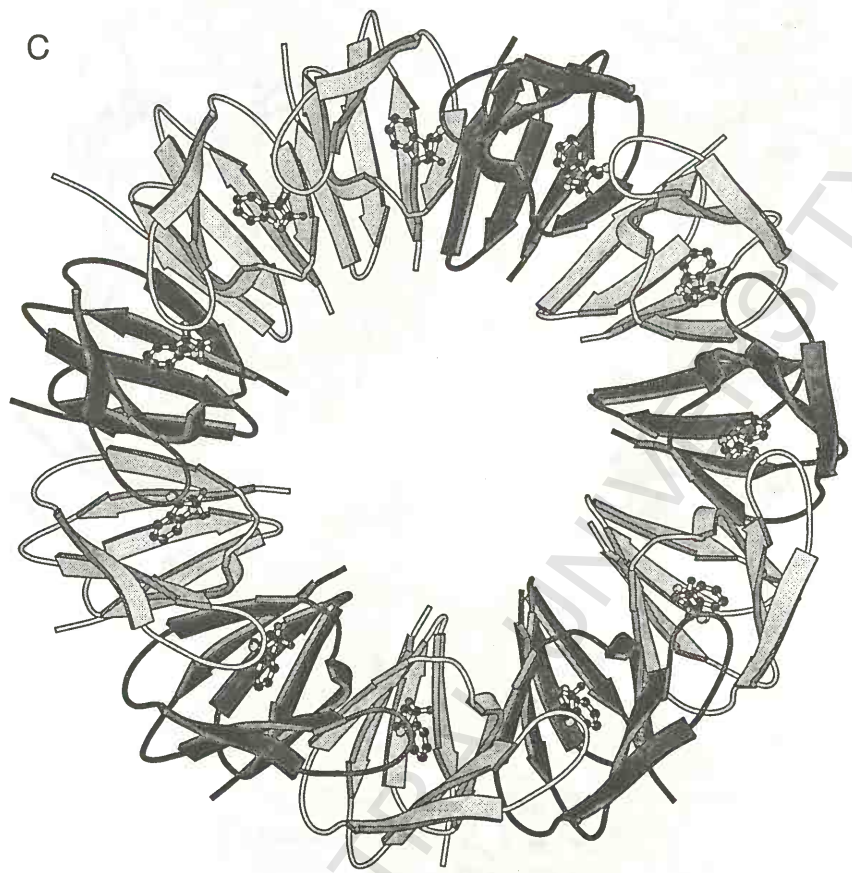

Figure 17.1.18 *(above and at left)* (**A**) The RNA-binding protein Rop from *E. coli.* (PDB entry 1rop). Helices 1 and 1′, postulated to bind to RNA, are labeled. (**B**) Hexamer of the MS2 phage-coat protein (PDB entry 1mst). Two AB dimers and a CC dimer are arranged around a quasi-3-fold rotation axis located in the center of the figure (labeled q3). (**C**) Eleven-subunit oligomer of Trp RNA-binding attenuation protein (TRAP; PDB entry 1wap). Bound tryptophan molecules are shown by a ball-and-stick model. Alternating monomers are shaded differently for clarity.

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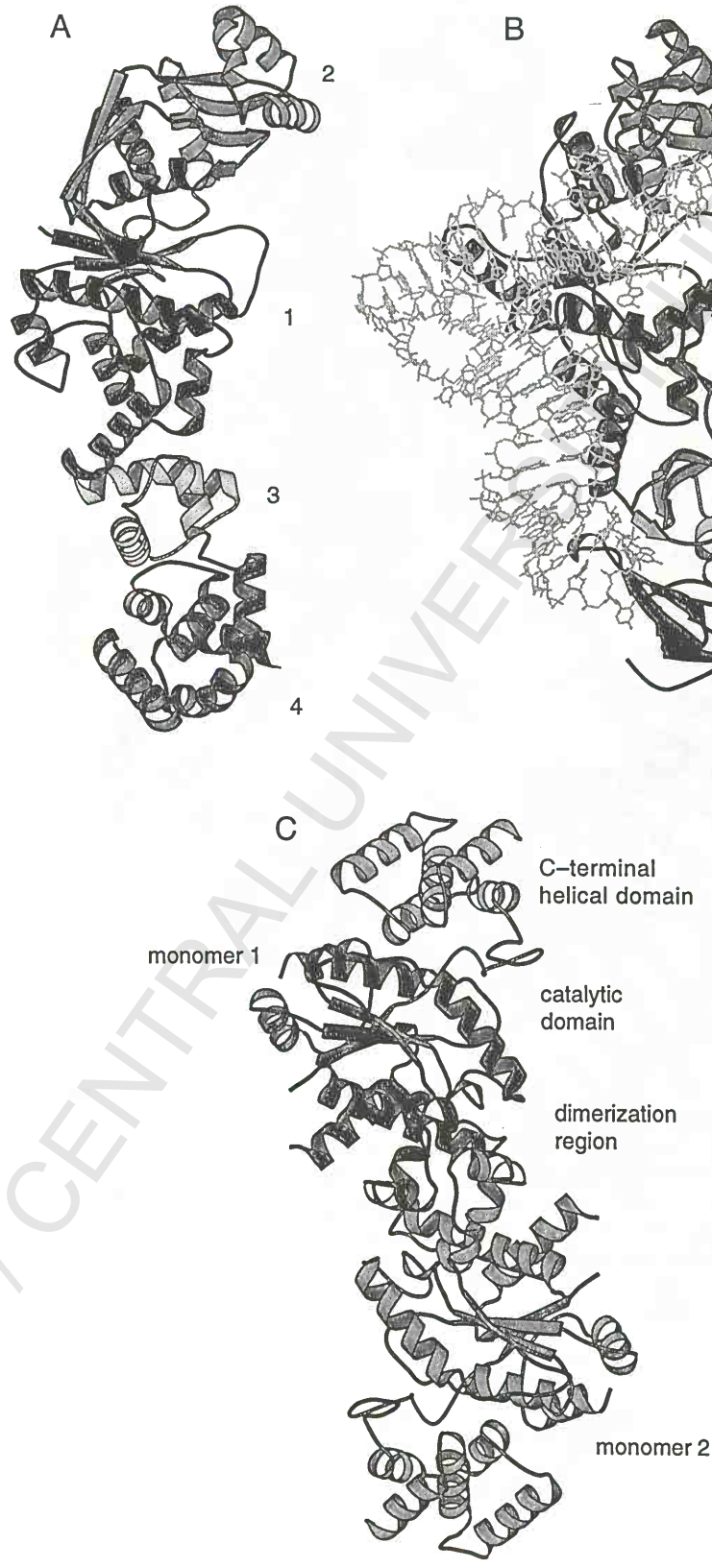

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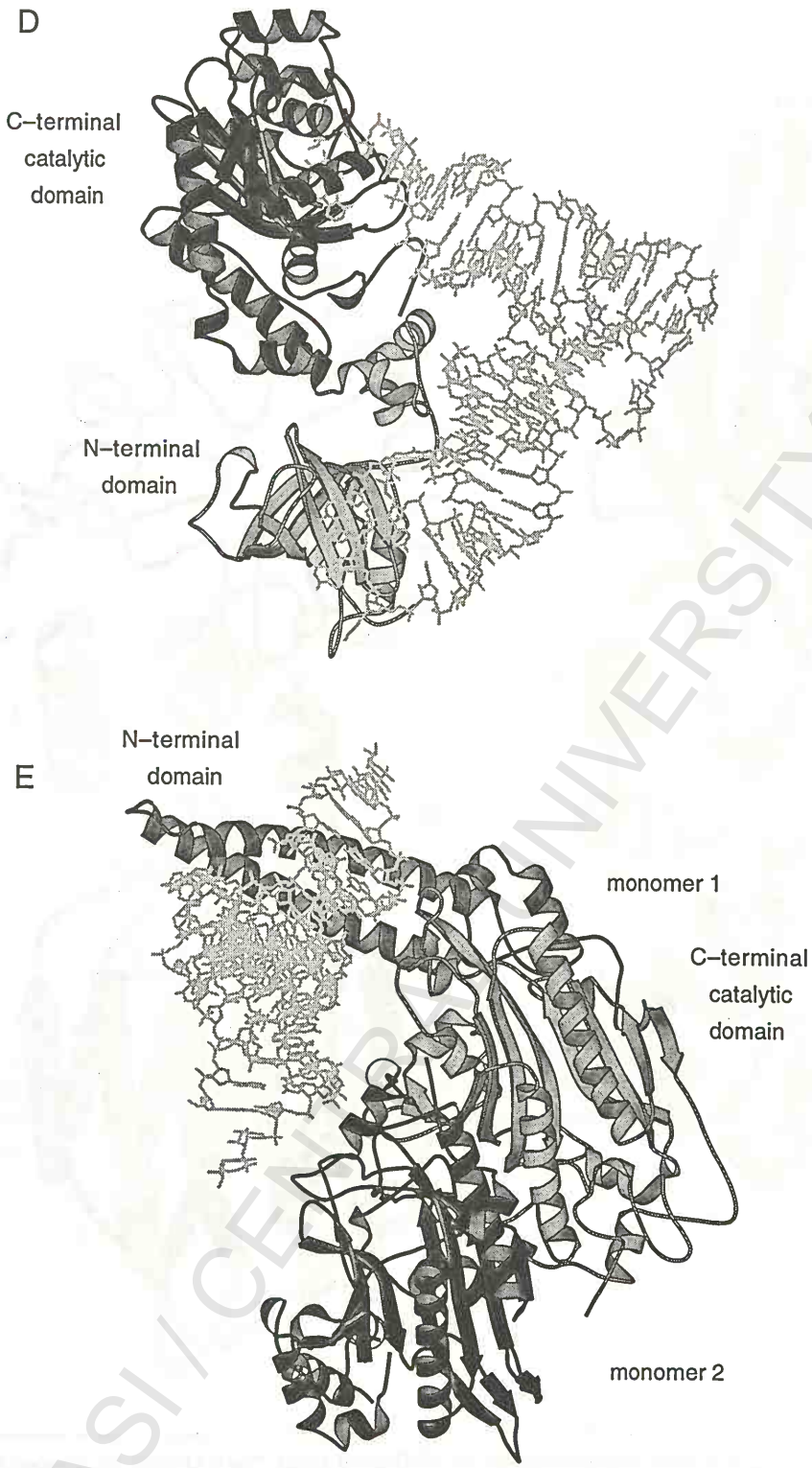

Figure 17.1.19 *(above and at left)* Class I and II tRNA synthetases. (**A**) Glutamyl-tRNA synthetase (class Ib; PDB entry 1gln). Domains 1 to 4 are labeled. (**B**) Glutaminyl-tRNA synthetase complexed with $tRNA^{Gln}$ (class Ib; PDB entry 1gtr). Domains 1 to 4 are labeled. (**C**) Functional homodimer of tyrosyl-tRNA synthetase (class Ic; PDB entry 1tyb). (**D**) Aspartyl-tRNA synthetase complexed with $tRNA^{Asp}$ class IIb; PDB entry 1asz). The N- and C-terminal domains are labeled. (**E**) Seryl-tRNA synthetase complexed with a fragment of $tRNA^{Ser}$ (class IIa; PDB entry 1ser). The N- and C-terminal domains of monomer 1 are labeled. The N-terminal domain of monomer 2 is not visible in the crystal structure.

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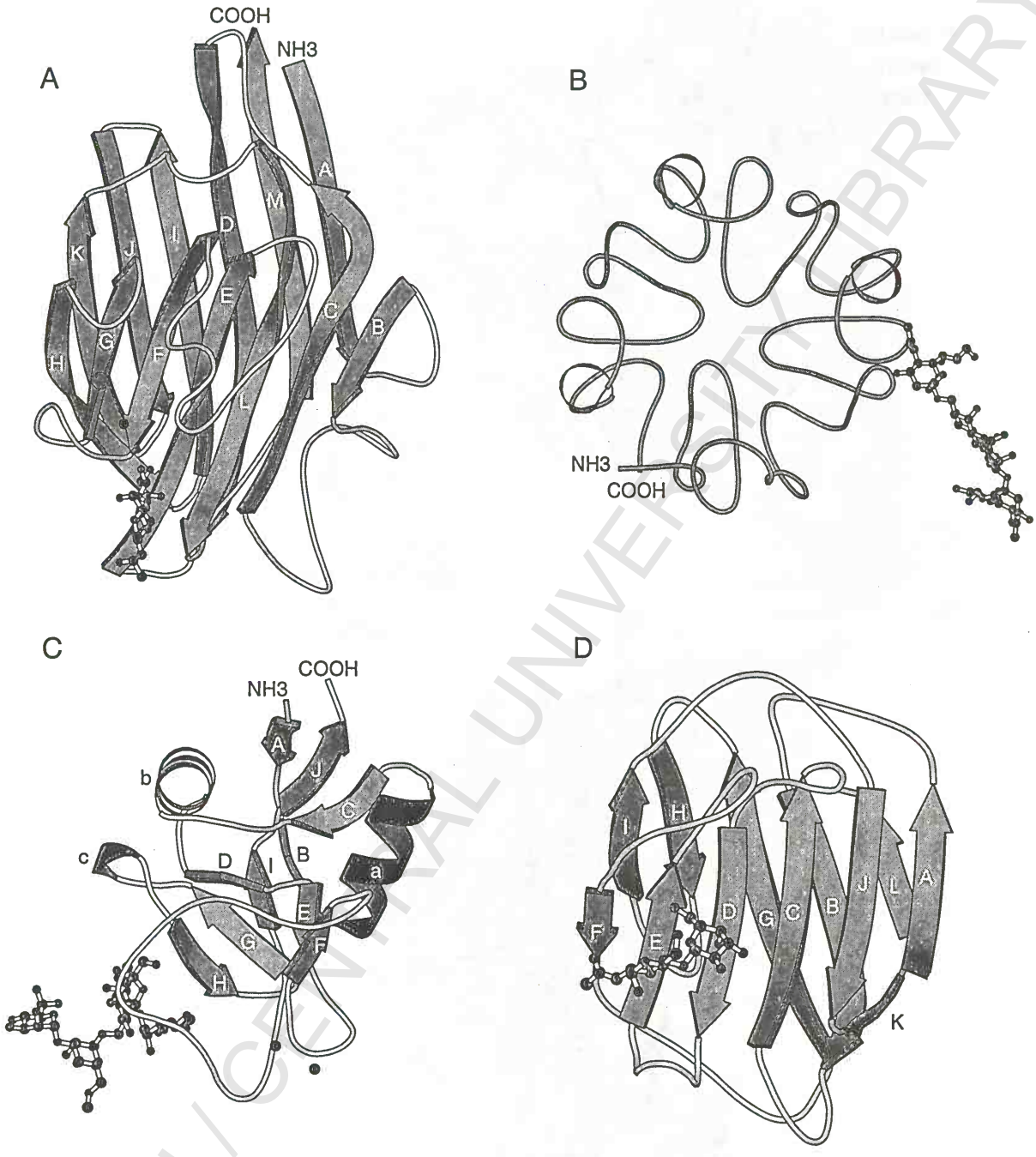

Figure 17.1.20 Lectin folds. (**A**) Legume lectin soybean agglutinin (PDB entry 1sba). The β strands are labeled A through M. The bound carbohydrate molecules are shown in ball-and-stick model. (**B**) Wheat-germ agglutinin monomer with bound carbohydrate (PDB entry 2wgc). (**C**) A C-lectin with bound carbohydrate (PDB entry 2msb). The helices are labeled a, b, and c. The β strands are labeled A to J. (**D**) S-lectin with its bound carbohydrate molecule (PDB entry 1slt).

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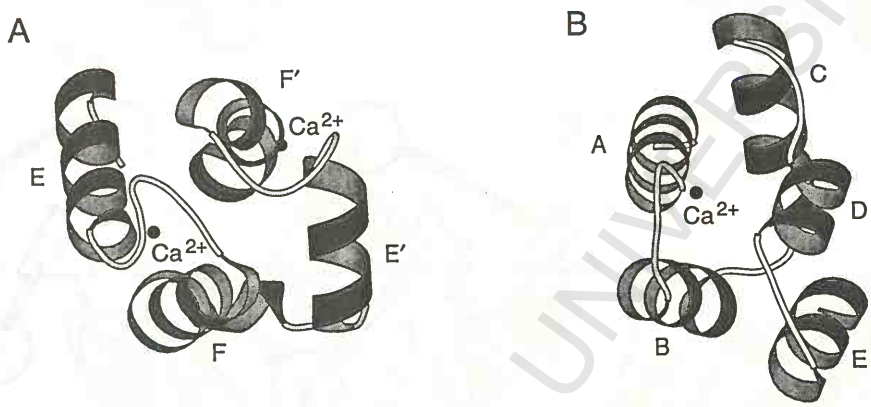

Figure 17.1.21 Calcium-binding folds. Calcium ions are drawn as solid spheres. (**A**) A pair of calcium-binding EF-hands complexed with Ca^{2+} from bovine calbindin D9K. The corresponding helices are labeled E, F, E′ and F′ (PDB entry 4icb). (**B**) Calcium-binding domain of annexin V (PDB entry 1ala).

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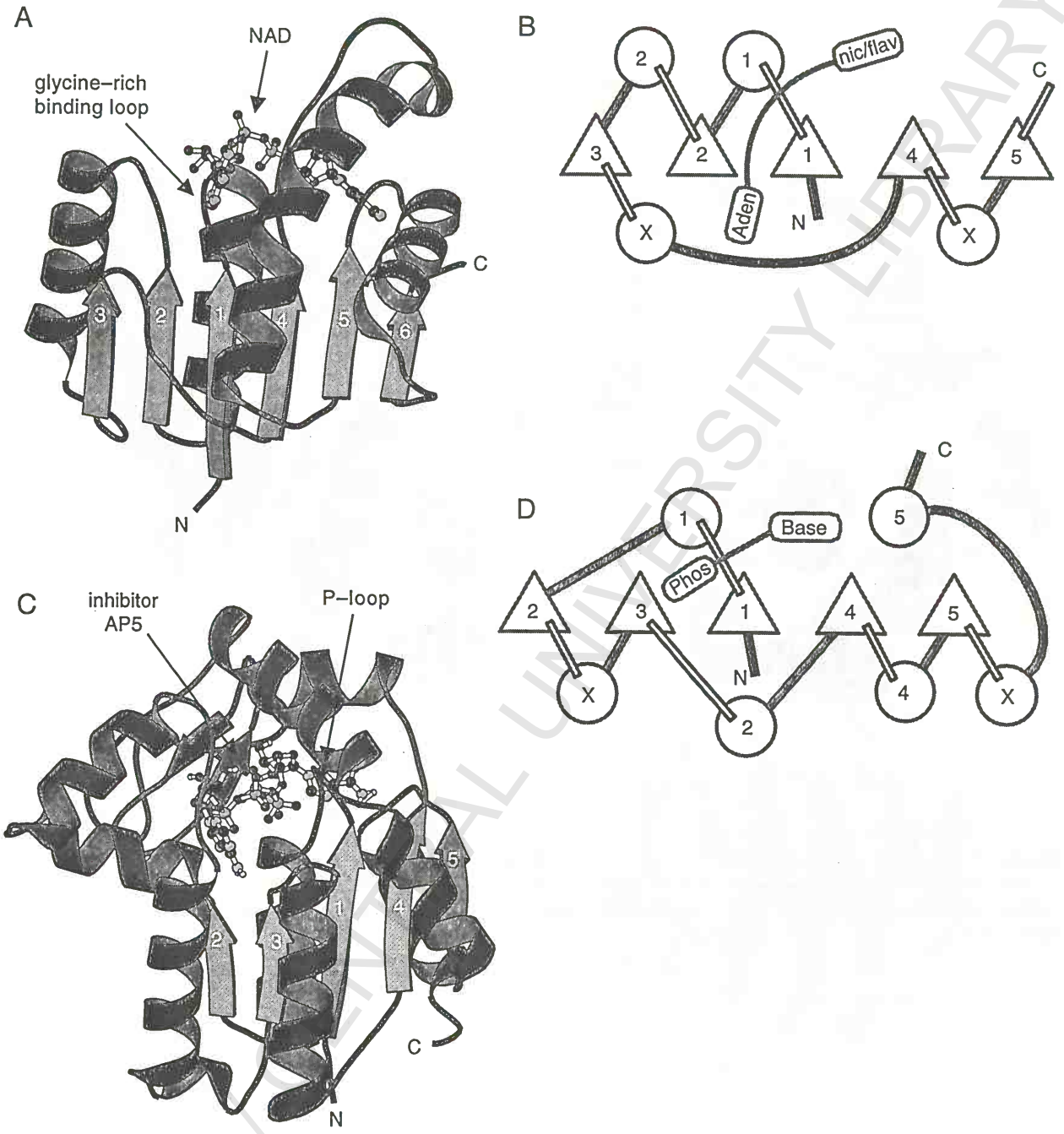

Figure 17.1.22 The classical dinucleotide- and mononucleotide-binding folds shown by ribbon drawings (left) and topology diagrams (right). In the topology diagrams (adapted from Schulz, 1992) the β sheets are viewed from the C-terminal edge with β strands represented by triangles and α helices by circles. Numbers indicate strand and helix order within the structure. Circles containing an "X" represent possible domain insertions. The bound nucleotides are dark. (**A**) Ribbon drawing of the NAD-binding domain of dogfish lactate dehydrogenase with bound NAD represented as a ball-and-stick model (PDB entry 1ldm). (**B**) Topology diagram for the common core of the classical dinucleotide-binding fold. (**C**) Ribbon drawing of adenylate kinase as an example of a mononucleotide-binding protein (PDB entry 1aky). The portion of the structure above the β sheet (four helices and a β hairpin) is not considered part of the classical mononucleotide-binding fold. The bound inhibitor *bis*(adenosine)-5′-pentaphosphate (AP5) is shown by a ball-and-stick model. (**D**) Topology diagram for the common core of the classical mononucleotide-binding fold.

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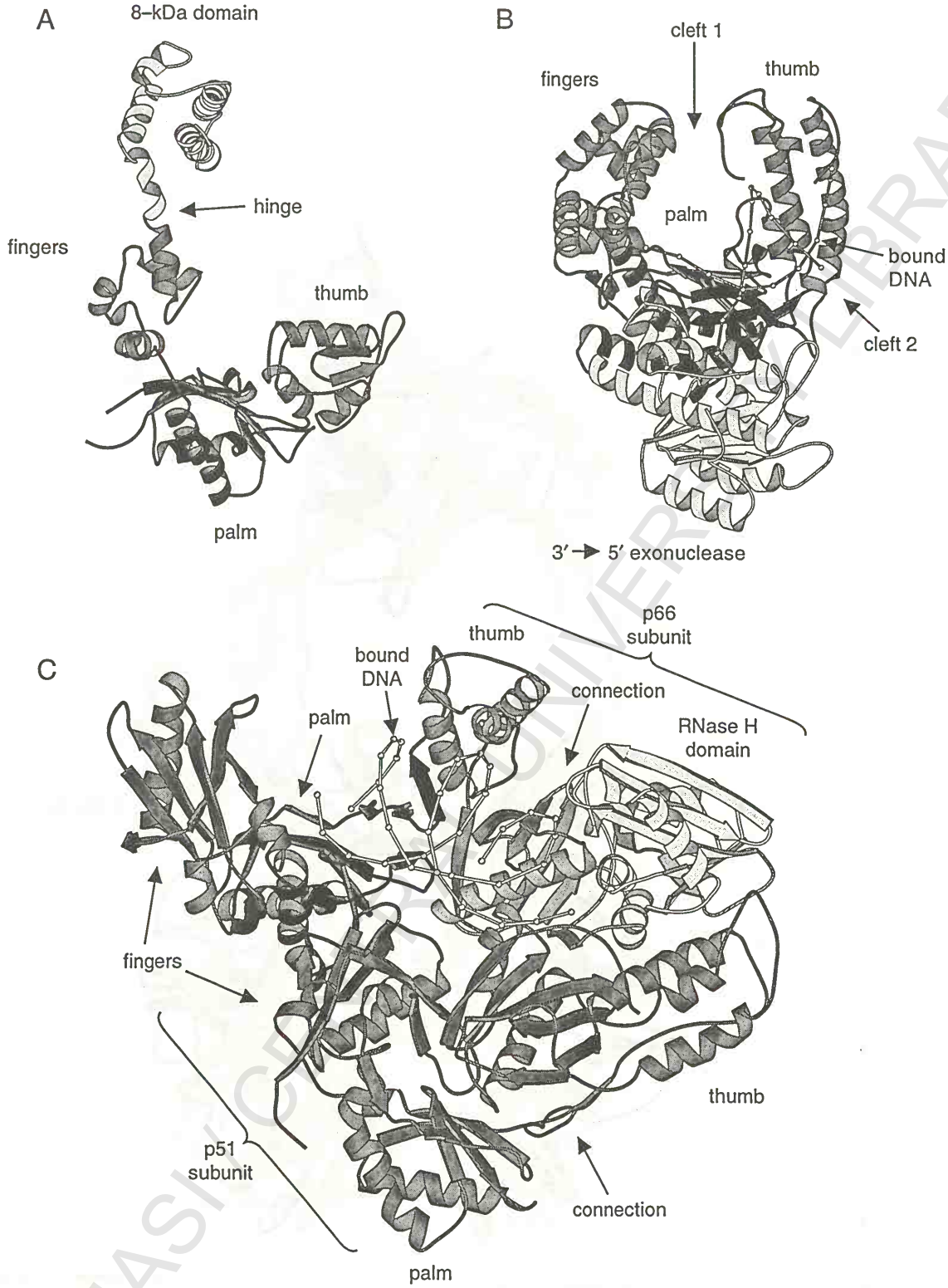

Figure 17.1.23 Structures of the catalytic domains of three DNA polymerases with the various subdomains labeled. (**A**) The 39-kDa catalytic domain of rat DNA polymerase β (PDB entry 1bpd). (**B**) The Klenow fragment of *E. coli* DNA polymerase I bound to eleven basepairs of duplex DNA in an editing complex (PDB entry 1kln). The bound DNA is represented by the light-shaded phosphate-backbone trace (labeled). (**C**) Structure of the HIV-1 reverse transcriptase heterodimer complexed with a 19/18-base duplex DNA (PDB entry 1hmi). The bound DNA is represented by a light-shaded phosphate-backbone trace (labeled).

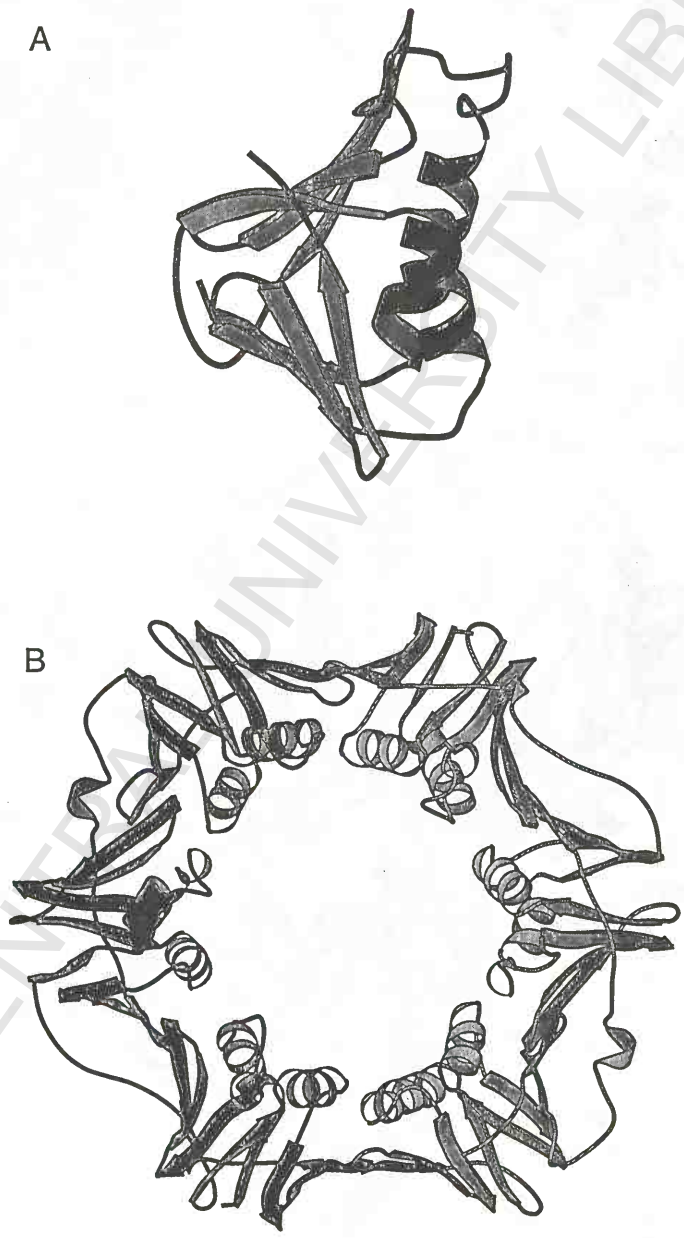

Figure 17.1.24 Structure of the β subunit homodimer of pol III (PDB entry 2pol). (**A**) A single domain of the β subunit. (**B**) View of the entire ring structure looking down the six-fold symmetry axis. The two monomers are shaded differently.

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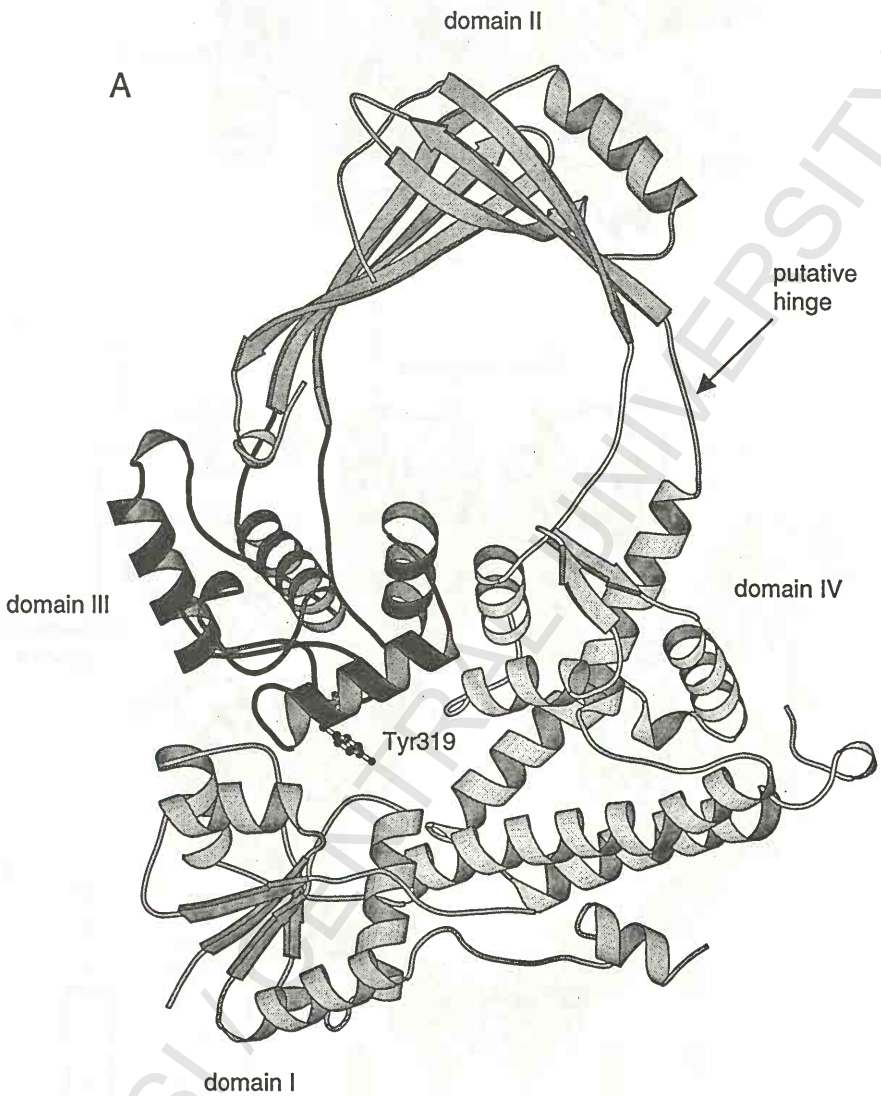

(continued on next page)

Figure 17.1.25 *(above and next page)* (**A**) Structure of the N-terminal 67-kDa fragment of *E. coli* topoisomerase I (PDB entry 1ecl). The four domains are labeled and the side chain of the active-site Tyr319 is represented by a ball-and-stick model. (**B**) Structure of the monomer of the 92-kDa fragment of yeast topoisomerase II (PDB entry 1bgw) showing the organization of domains. (**C**) Structure of the homodimer of topoisomerase II. The two monomers are shaded differently for clarity.

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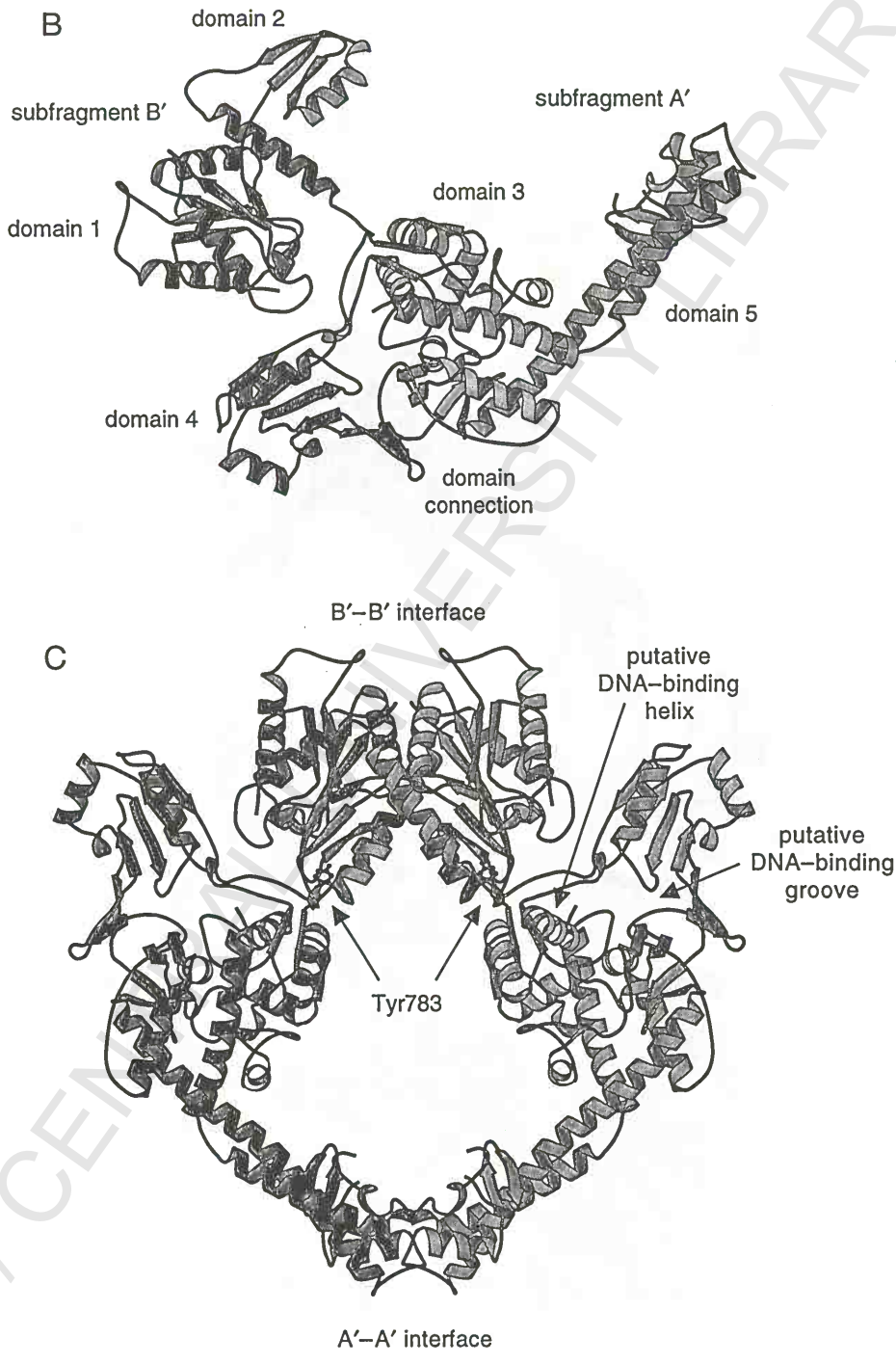

Figure 17.1.25 *(continued from previous page)*

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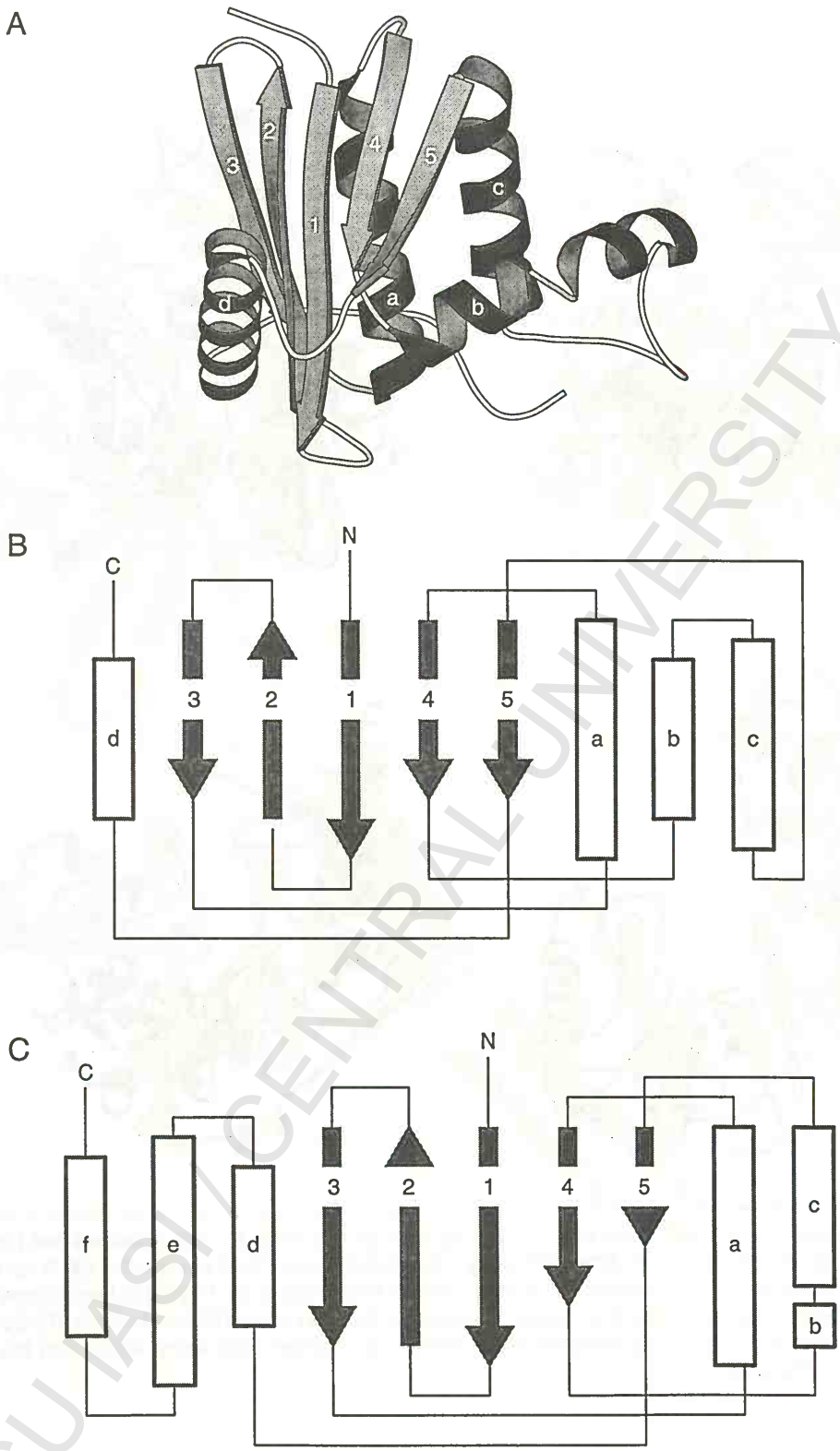

Figure 17.1.26 Members of the polynucleotidyl transferase fold family. (**A**) Structure of an RNase H from *E. coli* (PDB entry 2rn2). (**B**) Secondary structure topology diagram of Rnase H. (**C**) Secondary structure topology diagram of HIV integrase.

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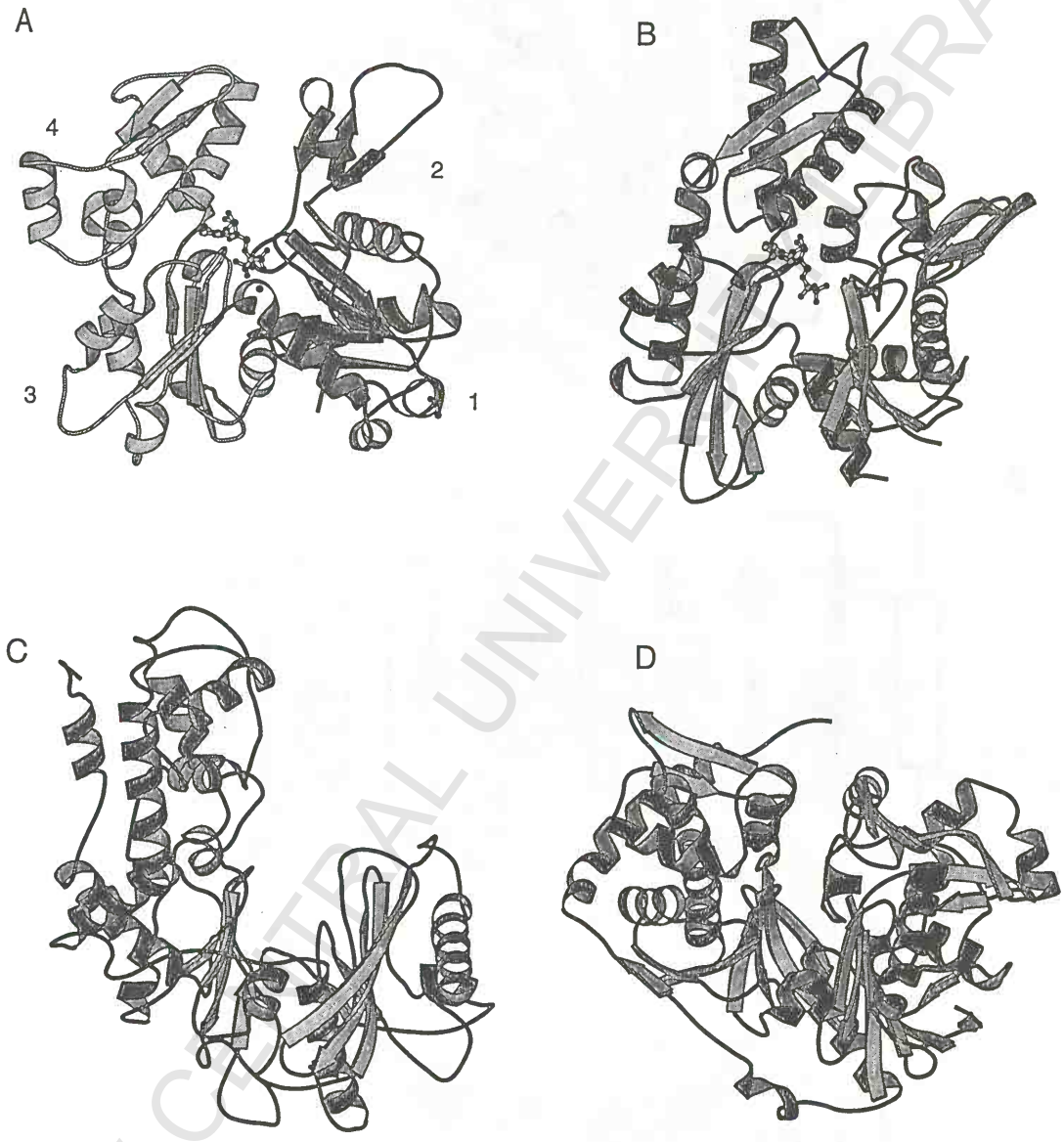

Figure 17.1.27 *(above and at right)* Structures containing the actin fold (**A** to **D**) and structures that bind to actin (**E** to **G**). (**A**) Actin complexed with ATP (PDB entry 1atn). Subdomains 1 to 4 are labeled. (**B**) N-terminal fragment of heat-shock cognate protein (hsc70) complexed with ADP (PDB entry 1nga). (**C**) Hexokinase B in its open form (PDB entry 2yhx). (**D**) Glycerol kinase (PDB entry 1gla). (**E**) Profilin (PDB entry 2btf). (**F**) Severin (NMR structure, PDB entry 1svq). (**G**) Spectrin repeat shown as a dimer (PDB entry 1spc), with the two monomers shaded differently.

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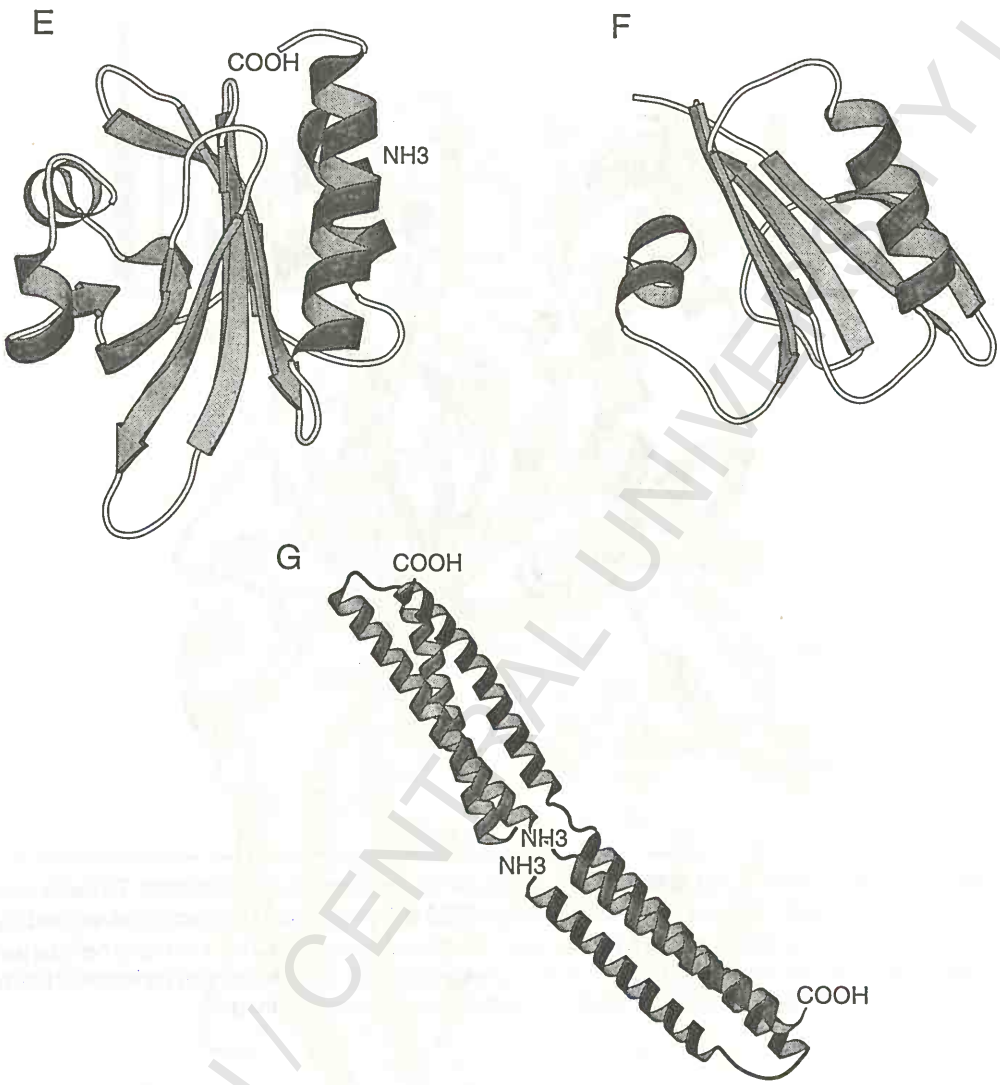

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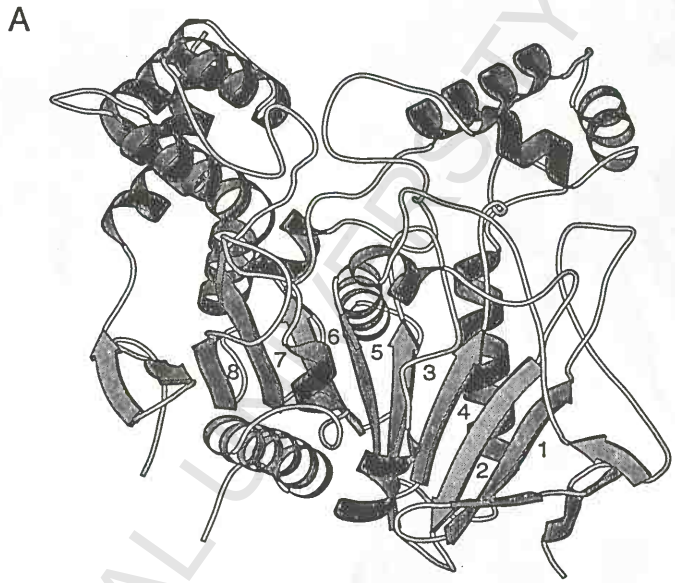

Figure 17.1.28 *(above and at right)* **(A)** Structure of the acetylcholinesterase from *Torpedo californica*. The central eight-stranded β sheet is shown (PDB entry 1ack). **(B)** Diagram of the secondary structure topology common to all α/β hydrolases. The strands are numbered 1 to 8 and helices are labeled A to F. **(C)** Structure of an influenza virus neuraminidase complexed with an inhibitor (PDB entry 1nnc). The active site–bound inhibitor is shown as ball-and-stick model.

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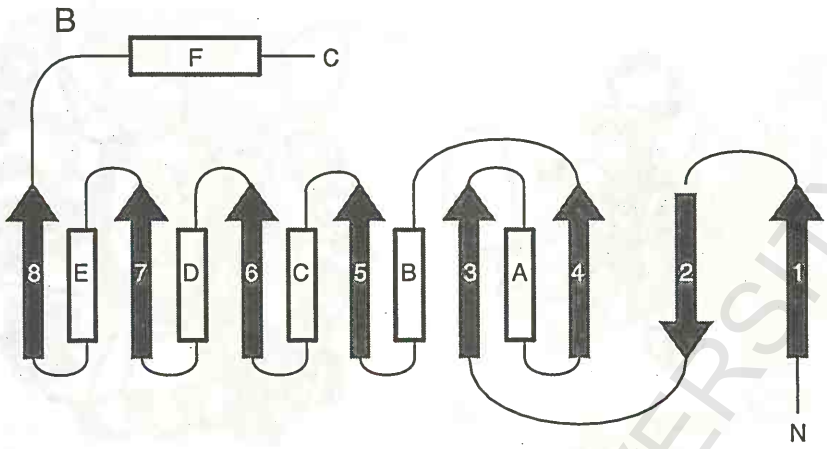

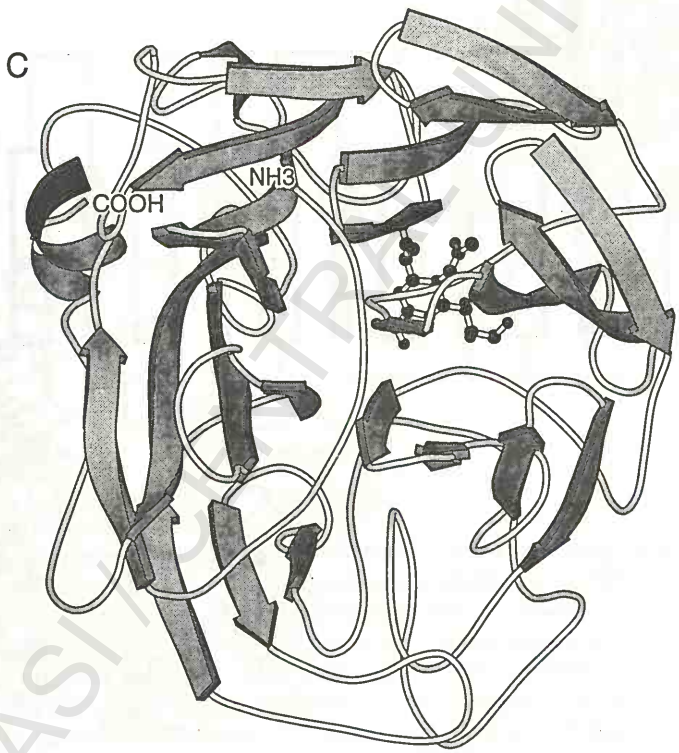

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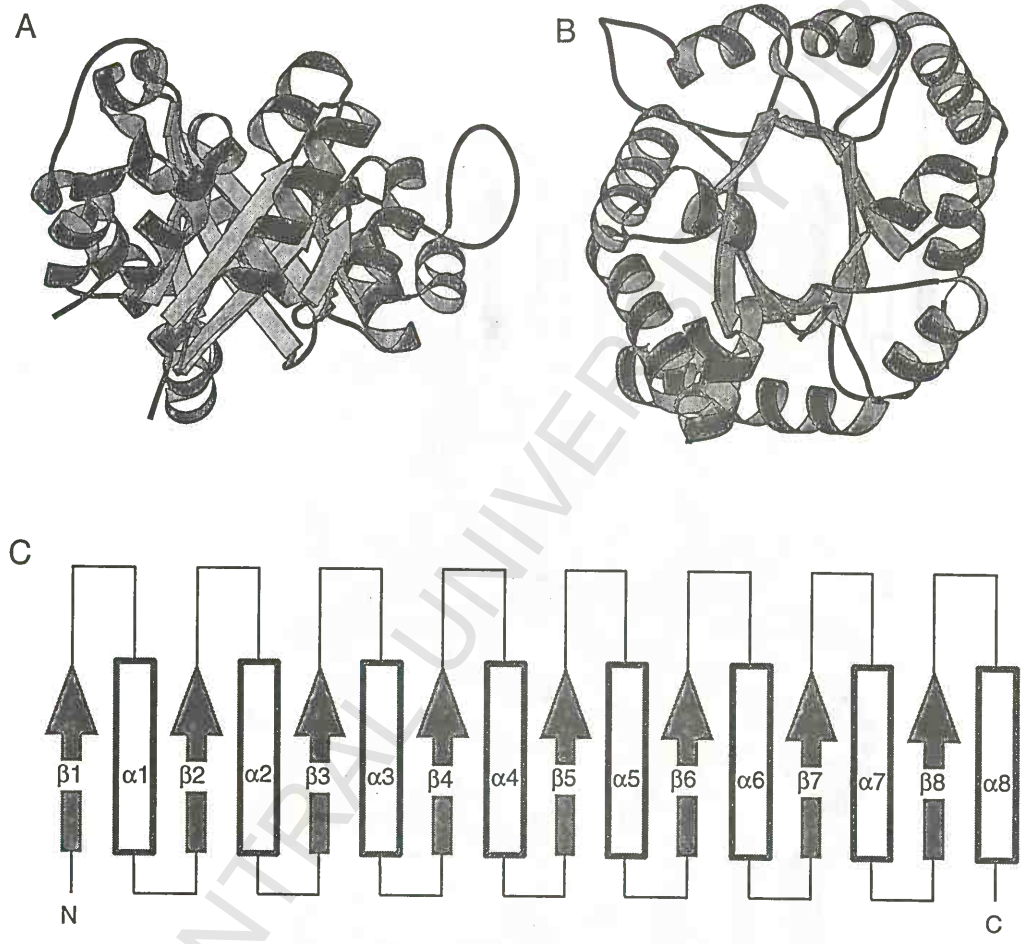

Figure 17.1.29 *(above and at right)* The TIM-barrel fold (**A** to **C**) and the serine protease folds (**D** to **F**). (**A**) Side view of a ribbon drawing of triose phosphate isomerase (PDB entry 1tim) as an example of a TIM barrel. (**B**) Top view of a ribbon drawing of triose phosphate isomerase. (**C**) Secondary-structure schematic of the classical TIM-barrel fold. β strands are represented by arrows and α helices by rectangles. (**D**) Collagenase (a trypsin-like serine protease; PDB entry 1hyl) with the side chains of residues that form the catalytic triad shown by a ball-and-stick model. (**E**) Subtilisin BPN′ (a subtilisin serine protease; PDB entry 1sup) with the side chains of residues that form the catalytic triad shown by a ball-and-stick model. (**F**) Wheat serine carboxypeptidase II (a serine carboxypeptidase; PDB entry 1wht) with the side chains of residues that form the catalytic triad shown by a ball-and-stick model.

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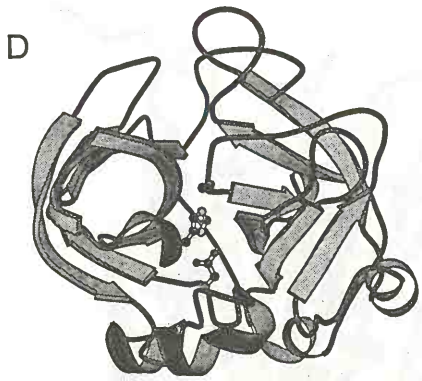

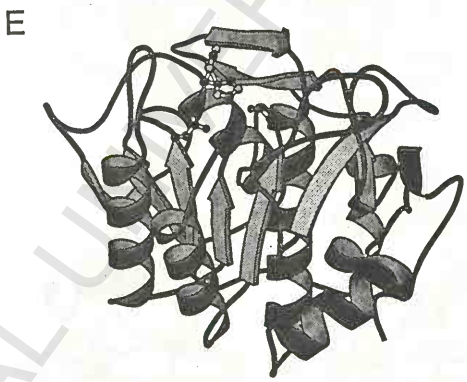

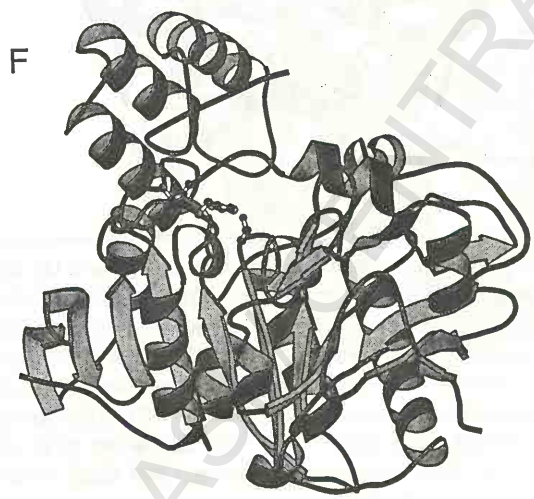

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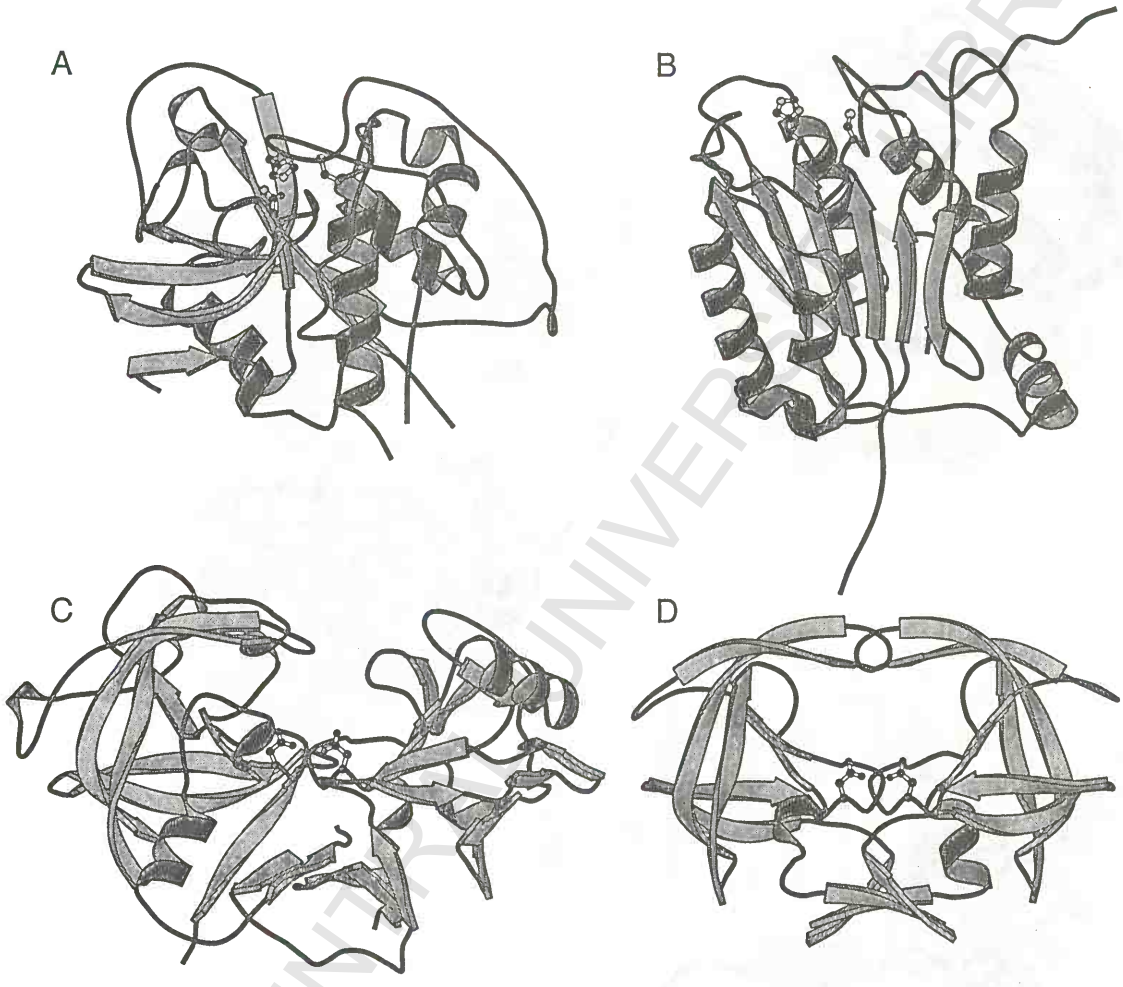

Figure 17.1.30 *(above and at right)* Examples of cysteine proteases (**A** and **B**), aspartic proteases (**C** and **D**), and metalloproteases (**E** to **G**). The side chains of active-site residues mentioned in the text are shown by ball-and-stick models. (**A**) Human cathepsin B (a papain-like cysteine protease; PDB entry 1huc). (**B**) Interleukin 1β–converting enzyme (ICE; PDB entry 1ice). (**C**) Human renin (a pepsin-like aspartic protease; PDB entry 1bbs). (**D**) HIV-1 protease (a retroviral aspartic protease; PDB 1hpx). (**E**) Snake venom adamalysin II (a zinc-dependent endopeptidase or zincin; PDB entry 1iag). (**F**) Aminopeptidase from *Aeromonas proteolytica* (a zinc-dependent exopeptidase; PDB entry 1amp). (**G**) Alkaline protease (a metzincin from *Pseudomonas aeruginosa*; PDB entry 1akl). The active-site zinc and coordinated side chains are shown as ball-and-stick models in the N-terminal catalytic domain (left side). Bound calcium ions are shown as balls in the C-terminal parallel β roll domain (right side).

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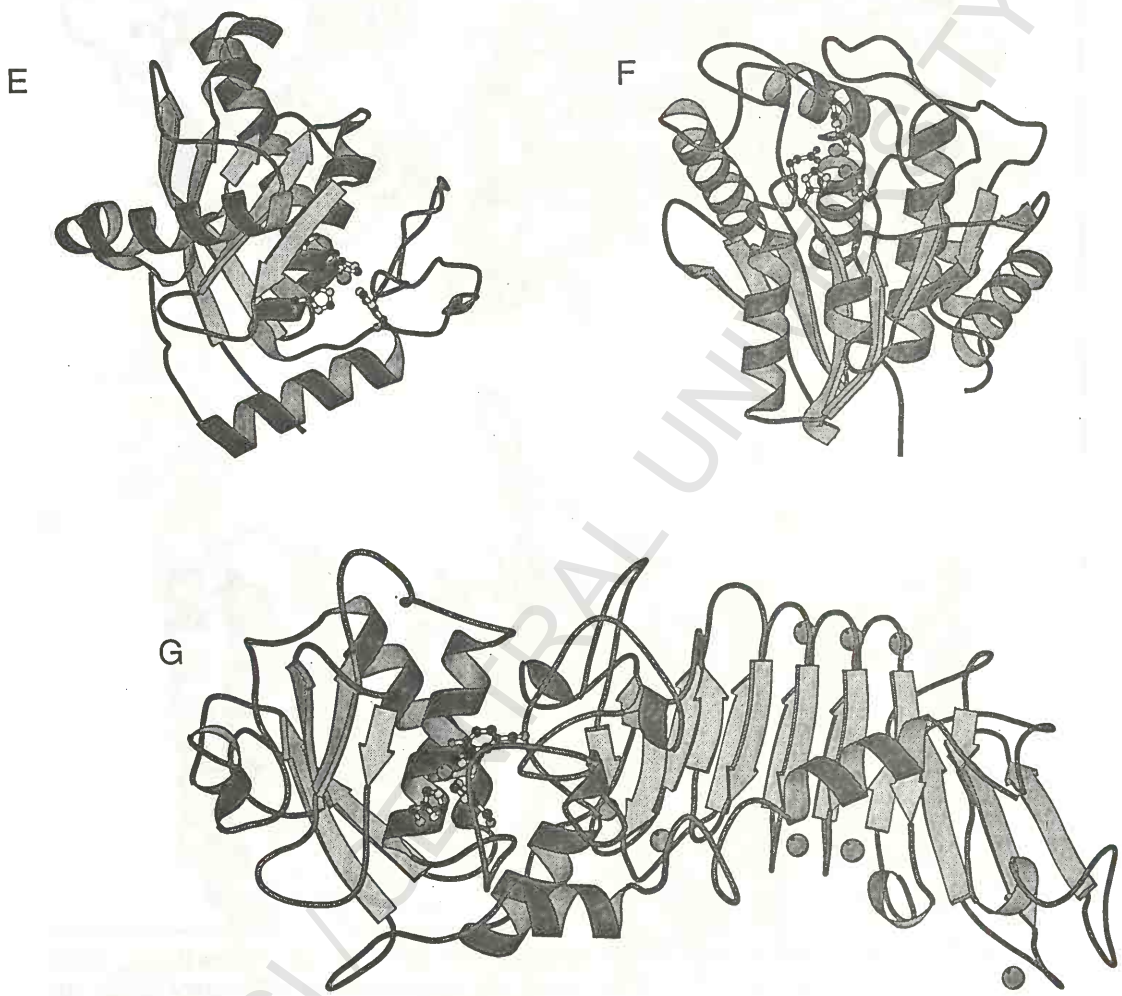

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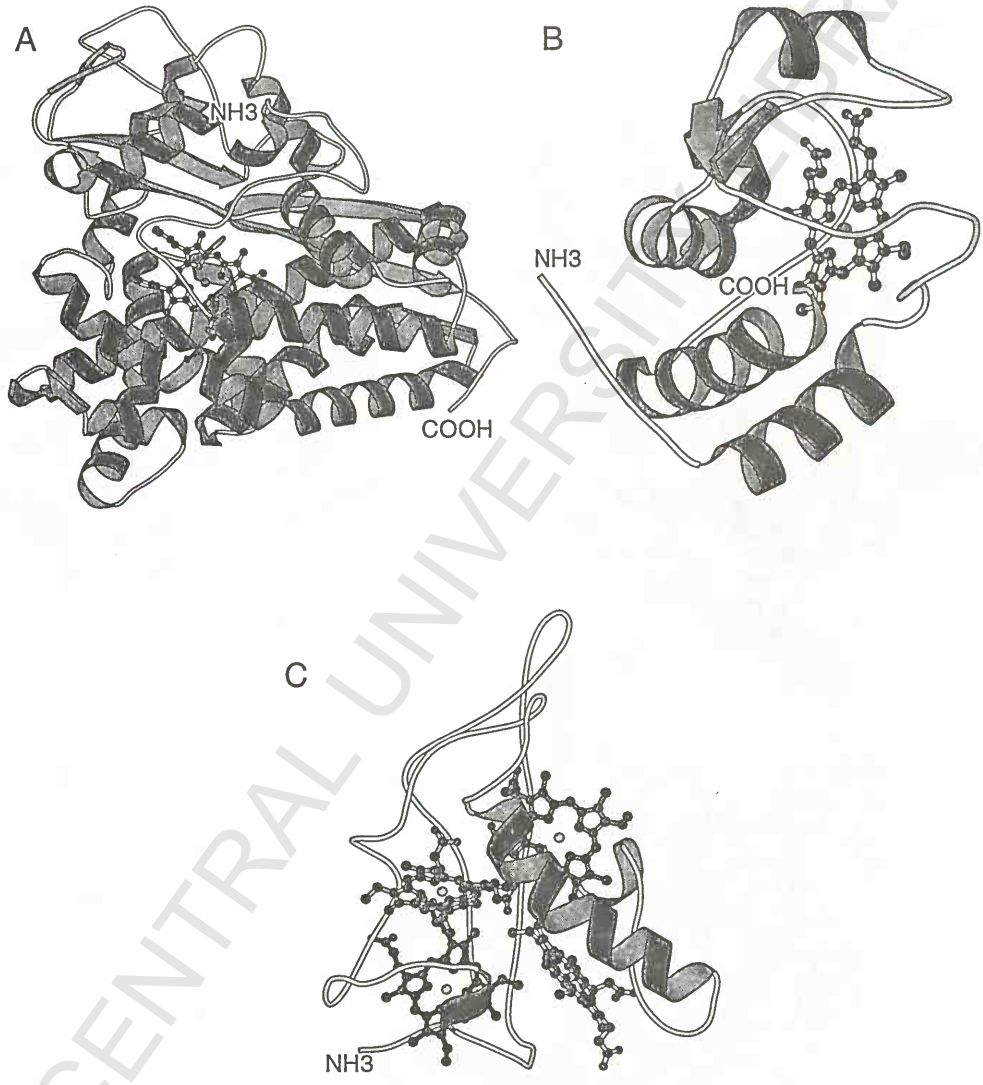

Figure 17.1.31 *(above and at right)* Structures showing the fold of (**A**) cytochrome P_{450cam} (PDB entry 1phc); (**B**) cytochrome *c* (PDB entry 1ycc); (**C**) cytochrome *c3* (PDB entry 2cy3); (**D**) cytochrome *b5* (PDB entry 1cyo); (**E**) cytochrome *b562* (PDB entry 1cgn); and (**F**) a dimer of bacterioferritin (also known as cytochrome *b1*; PDB entry 1bcf) with the hemes shown by ball-and-stick models.

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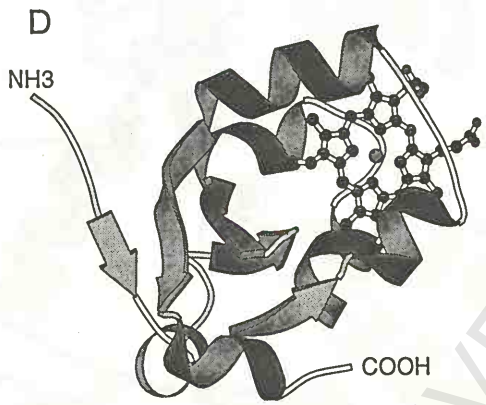

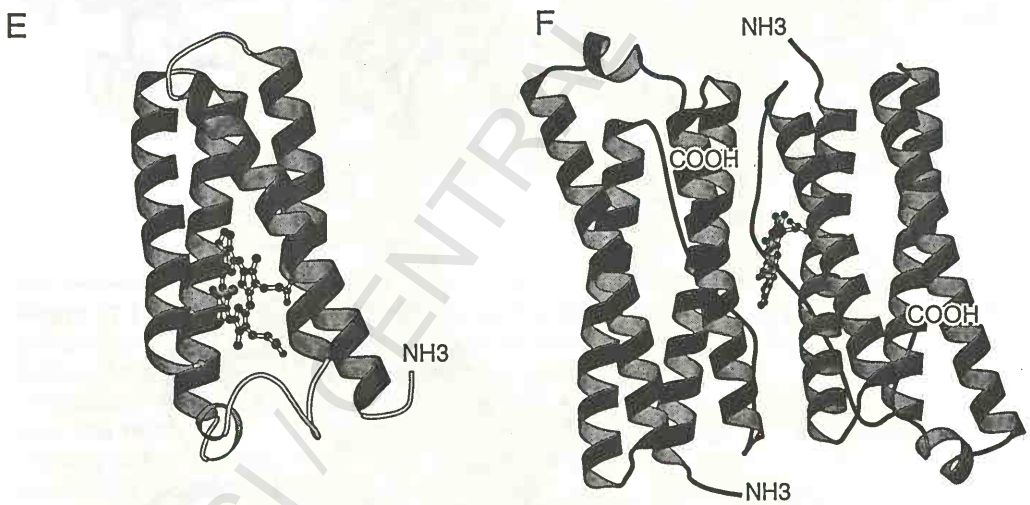

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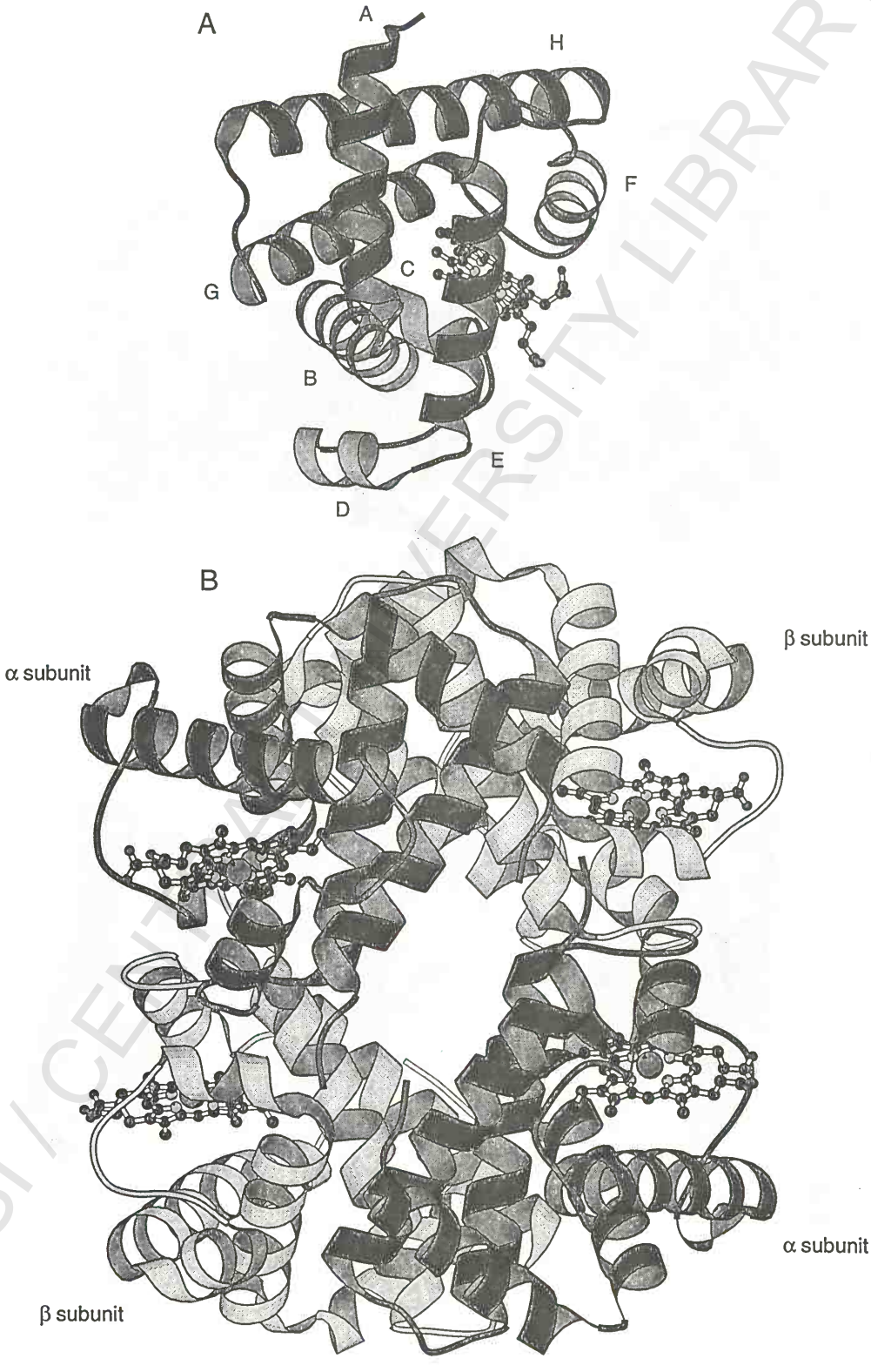

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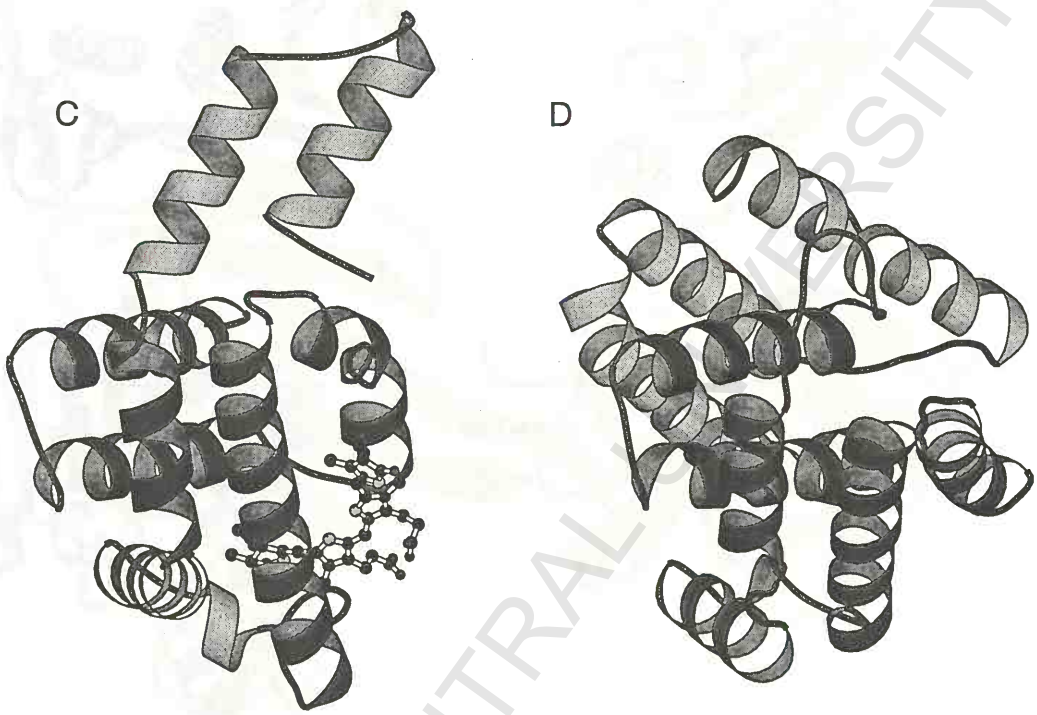

Figure 17.1.32 *(above and at left)* Structures of globin-like proteins from each of the three globin-like families. Panels A, C, and D are shown in the same orientation with respect to the globin fold. Helices that are not considered part of the core globin fold are shaded more lightly. **(A)** Sperm whale myoglobin (PDB entry 1mbd). Helices are labelled A to H according to traditional globin nomenclature. The heme group is represented by a ball-and-stick model. **(B)** Human deoxyhemoglobin, showing all four subunits (PDB entry 1hhb). The heme groups are represented by ball-and-stick models. **(C)** C-phycocyanin from cyanobacteria (PDB entry 1cpc). The phycocyanobilin cofactor is represented by a ball-and-stick model. **(D)** Colicin A from *E. coli* (PDB entry 1col).

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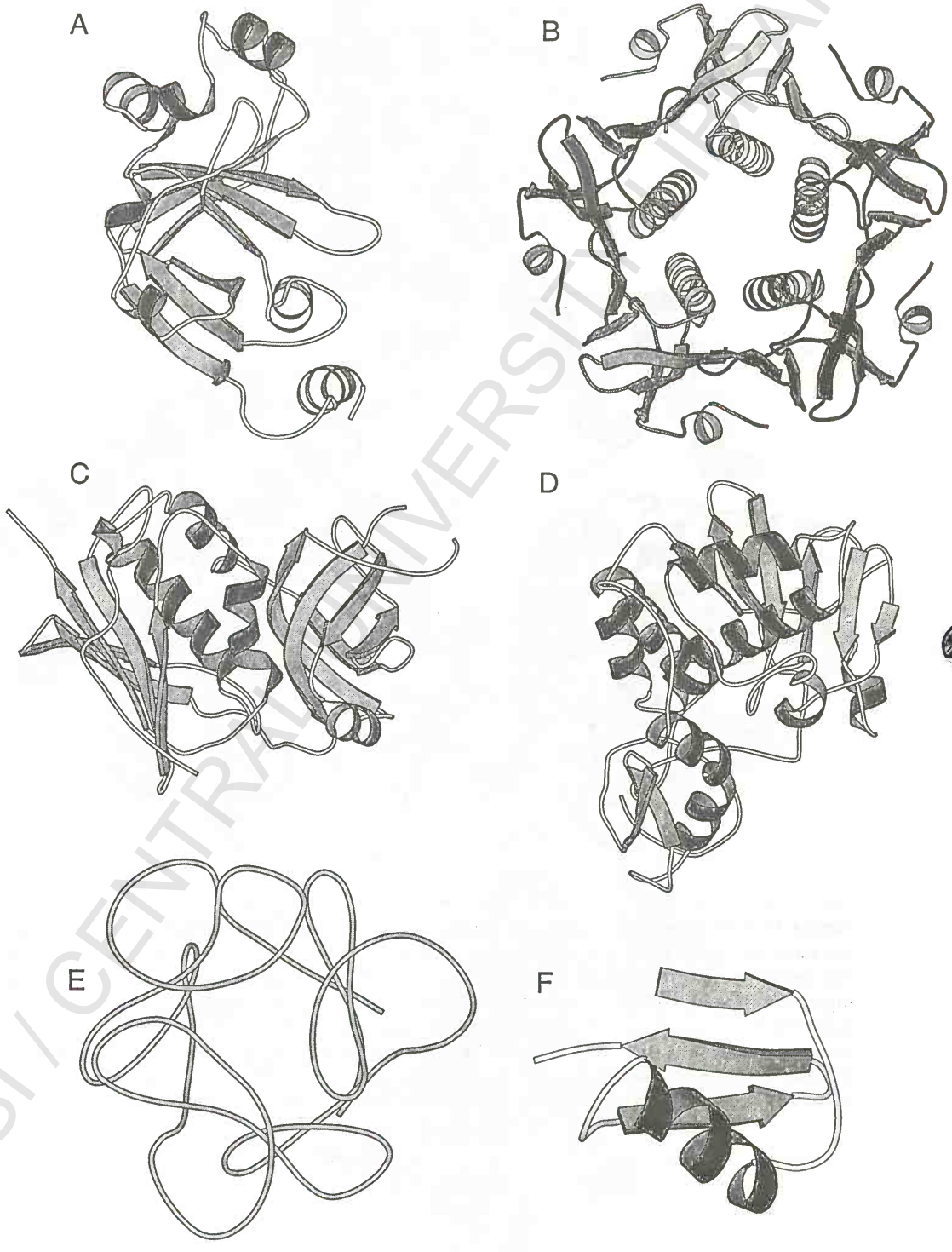

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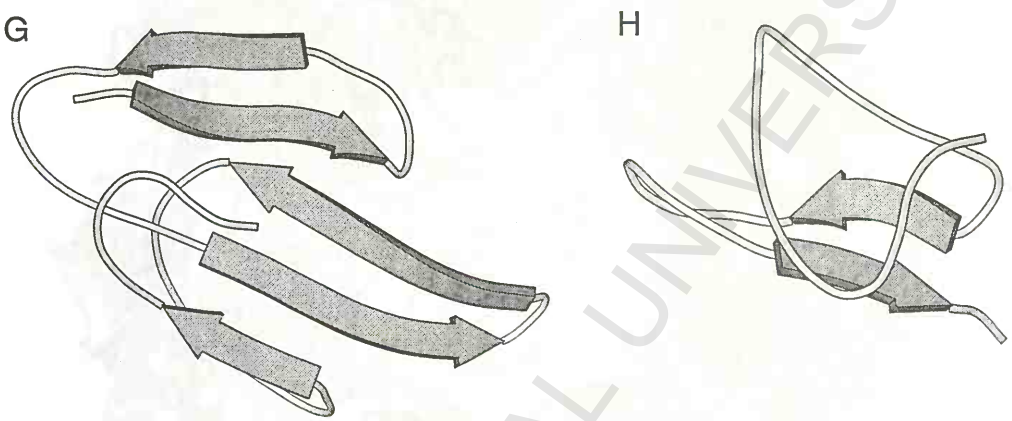

Figure 17.1.33 *(above and at left)* Tertiary folds of toxins. (**A**) ADP ribosylation domain of diphtheria toxin (PDB entry 1ddt). (**B**) Pentameric B domain of bacteria AB5 cholera toxin with each domain shaded differently (an ADP ribosylation toxin; PDB entry 1chp). (**C**) Superantigen toxin (PDB entry 1se2). (**D**) Ricin A chain and (**E**) ricin B chain (from a ribosome-inactivating toxin; PDB entries 1rtc and 2aai, respectively). (**F**) Scorpion toxin (a small protein toxin; PDB entry 1mtx). (**G**) Snake toxin (a small protein toxin; PDB entry 1nxb). (**H**) Spider toxin (a small protein toxin; PDB entry 1eit).

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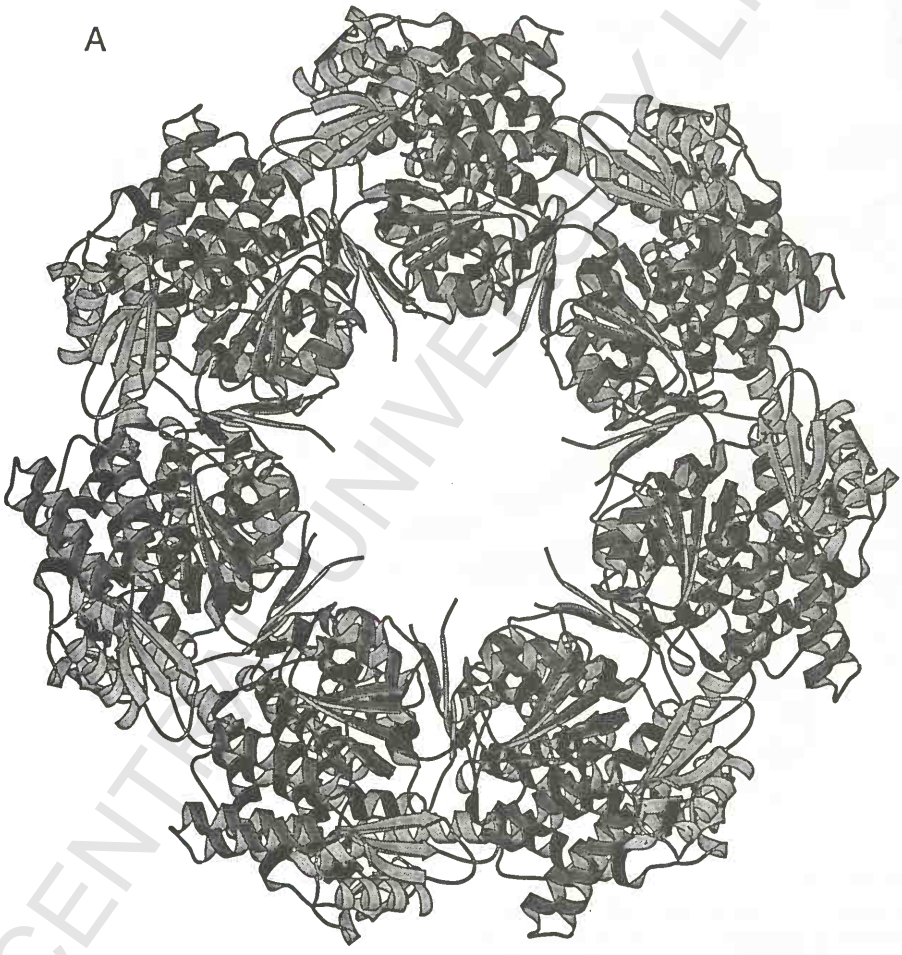

Figure 17.1.34 *(above and at right)* Chaperonin structures. (**A**) The GroEL heptameric ring (PDB entry 1oel). (**B**) Cα trace of GroES (PDB entry 1lep). (**C**) A GroEL monomer.

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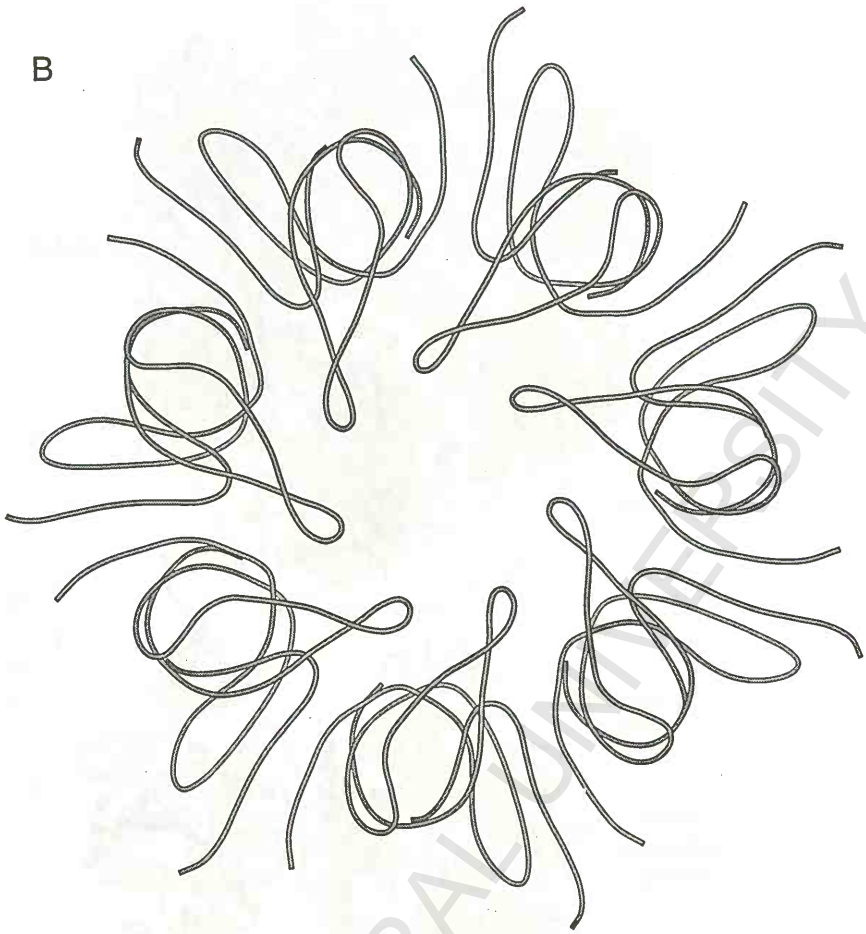

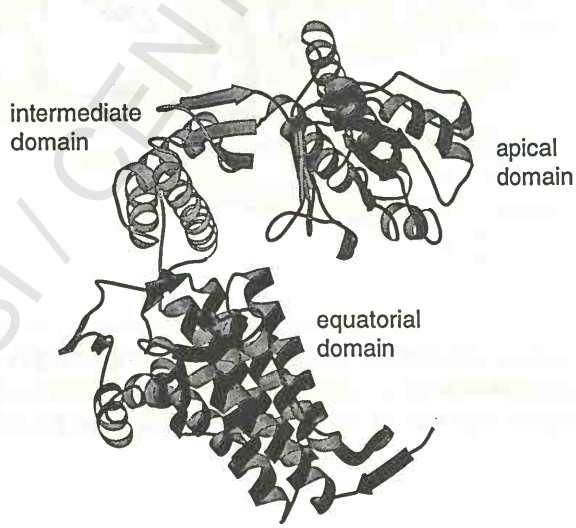

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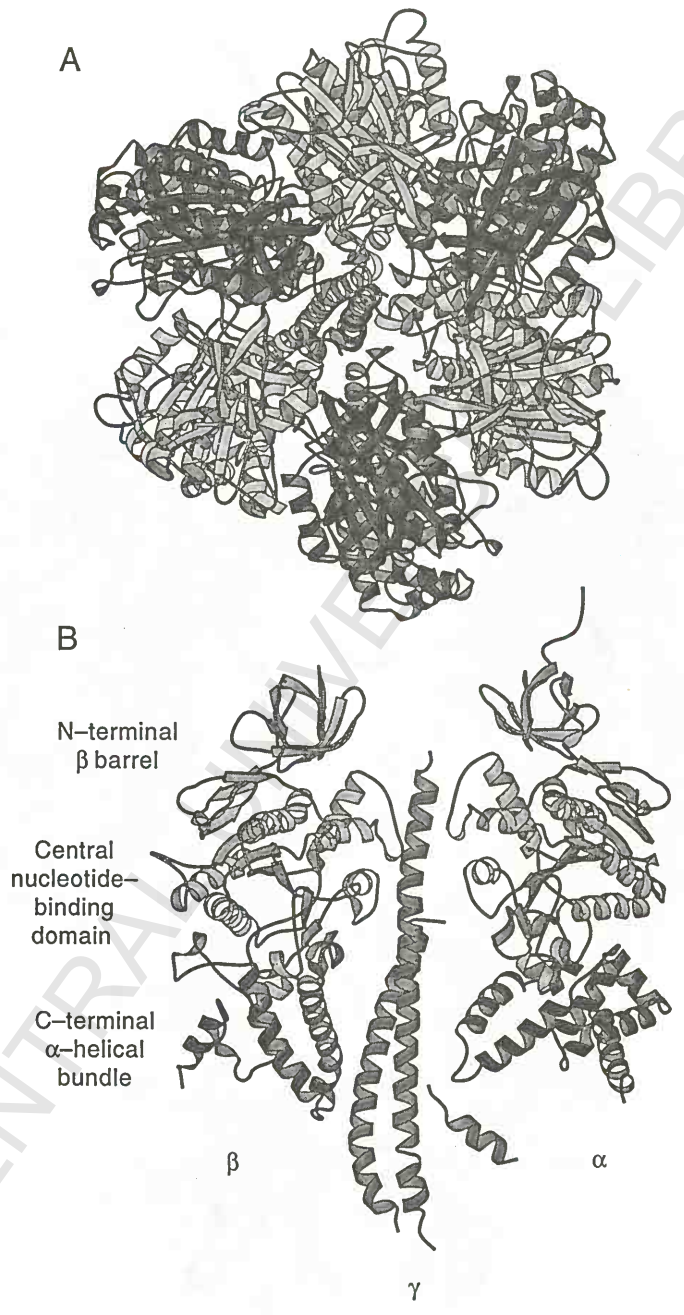

Figure 17.1.35 Structure of bovine heart mitochondrial F_1-ATPase (PDB entry 1cow). (**A**) Top view of the entire crystal structure including $\alpha_3\beta_3\gamma$ of F_1-ATPase. The α and β subunits are shaded light and dark, respectively. (**B**) Side view of one α subunit (left side), one β subunit (right side), and the γ subunit (center).

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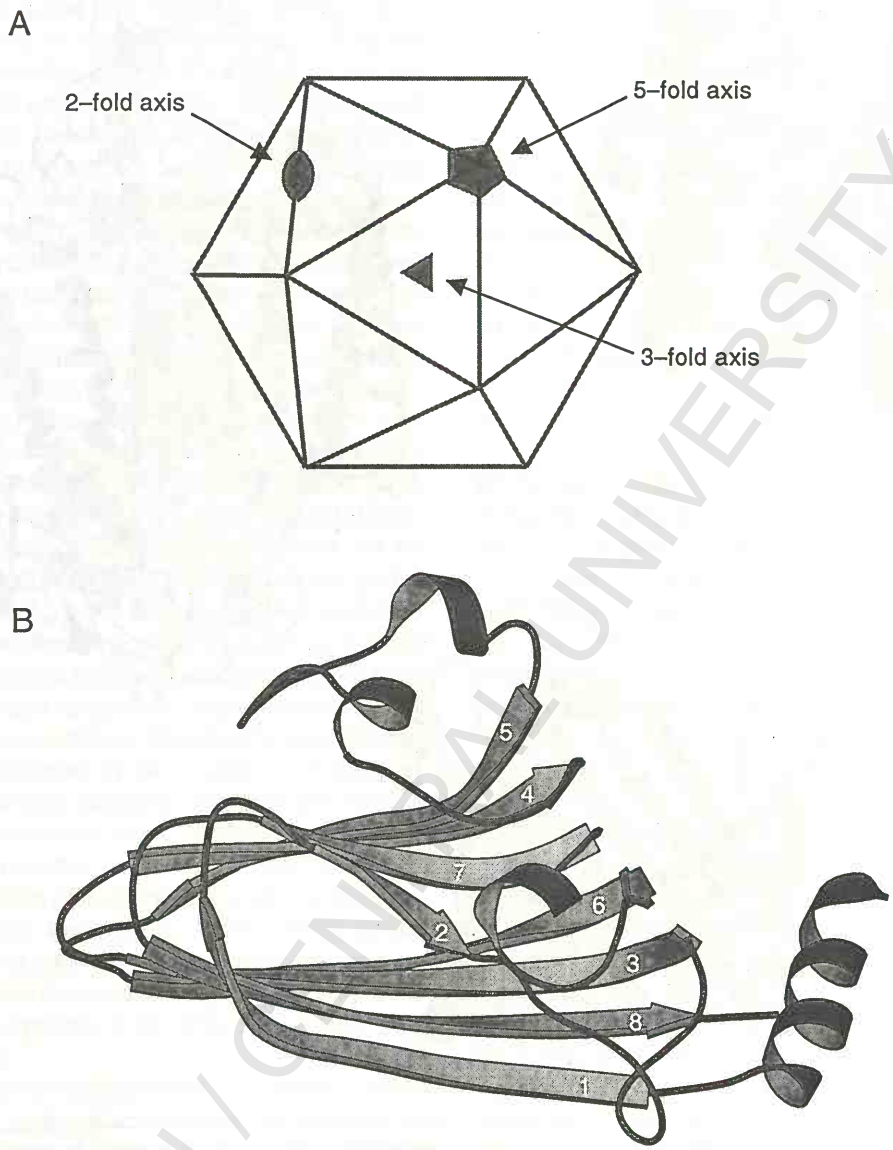

Figure 17.1.36 Viral capsid proteins. (**A**) Illustration of an icosahedron showing 2-fold, 3-fold, and 5-fold symmetry. (**B**) Structure of the jelly-roll β-sandwich fold of satellite tobacco necrosis virus-coat protein (PDB entry 2stv).

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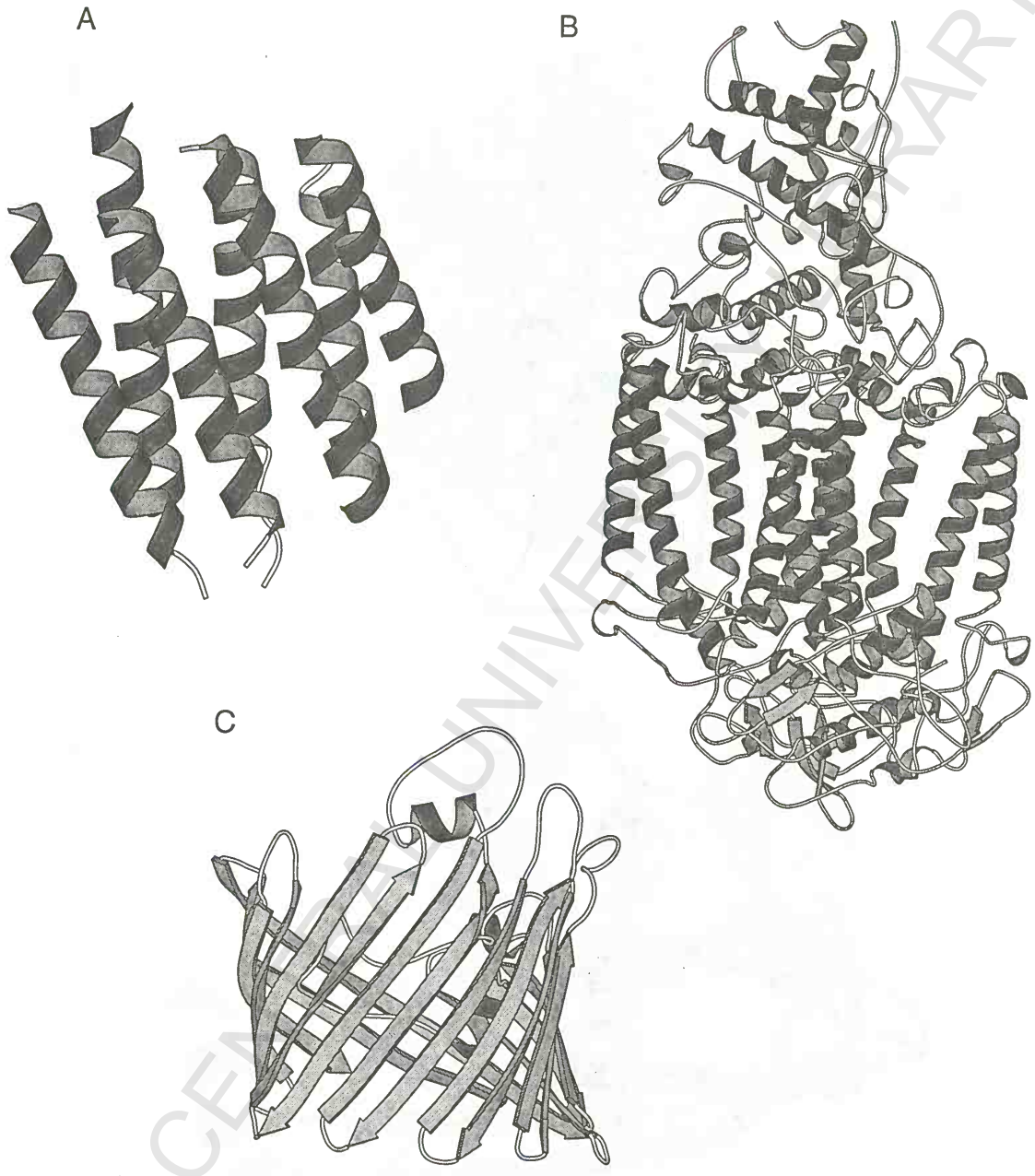

Figure 17.1.37 Examples of structures of integral membrane proteins. (**A**) Bacteriorhodopsin (PDB entry 1brd). (**B**) Photosynthetic reaction center (PDB entry 1prc). (**C**) Porin (PDB entry 2por).

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Overview of Macromolecular Electron Microscopy: An Essential Tool in Protein Structural Analysis

Although the majority of biological studies employing the electron microscope (EM) aim to visualize the substructure of cells and tissues or the spatial distributions of specific protein antigens in them, EM also has a long and distinguished record in the study of isolated protein molecules. Today, its potentialities for incisive observations of proteins and interactions between them are unprecedentedly high. These exciting prospects stem from innovations in specimen-preparation techniques, image-recording procedures, and importantly, advances in digital image processing (Baumeister and Zeitler, 1992; Frank, 1996). Of particular note, EM lends itself well to the examination of proteins or protein complexes that are refractory to other experimental approaches—e.g., proteins that are exceptionally large, flexible, filamentous or otherwise asymmetric, membrane-bound, or irremediably heterodisperse.

EM is an underutilized resource for the study of proteins in general, and one goal of this unit is to illustrate why EM is a powerful tool at the protein scientist's disposal. A second objective is to illustrate the potential of EM for structural analysis of large, multicomponent protein complexes. The significance of this area is heightened by the consideration that many, if not most, important biological processes are carried out by such complexes, not by simple enzymes that communicate only via random diffusion of their reaction products. DNA replication, transcription, RNA splicing, protein folding, protein synthesis, regulatory proteolysis, membrane trafficking, and signal transduction represent a few examples of such processes.

EM is complementary to—not in competition with—other methods for studying the structures of proteins. In that context, this unit will try to identify situations in which EM may offer the best, if not the only, prospect of progress—as well as situations where a combined approach in which EM is integrated cohesively with other techniques may be advantageous. Although a few atomic models of proteins have now been worked out by EM (see discussion of High-Resolution Studies) and more are on the way, it seems inevitable that the majority of new high-resolution structures will continue to emerge from X-ray crystallographic and NMR spectroscopic studies. One of the major opportunities that EM offers is in providing a means to capitalize on this hard-won information on the structures of individual subunits. In other words, EM has the potential to visualize how such subunits interact in higher-order, functional complexes.

The goals of this chapter are to give a general overview of current EM procedures for observing proteins, to outline the kind of information that may be acquired, and to illustrate their potential with some specific examples. The references represent an eclectic mix of reviews, early influential papers on given topics, accounts of methodology, and examples of recent or notably successful applications.

ELECTRON MICROSCOPY AND DIGITAL IMAGE PROCESSING: A PERFECT PARTNERSHIP

Electron micrographs of proteins tend to be noisy images, even when recorded with the most advanced instruments. Moreover, they provide only two-dimensional representations, usually projections, of specimens whose three-dimensional structures are the objects of primary interest. Both of these drawbacks have been largely eliminated by the emergence of digital image processing techniques specifically designed for analyzing micrographs of biological specimens (Misell, 1978; Frank, 1996). These techniques clarify and quantify the information recorded in the original micrographs. For instance, one may average many noisy images to obtain a better defined and more readily interpretable image of a protein, synthesize multiple views into a three-dimensional density map, or apply a variety of visualization tools to explore these maps and averaged two-dimensional images. In consequence, the scope of EM for studies of proteins has been immensely enhanced.

In contrast to X-ray crystallography, in which most practitioners tend to use the same highly developed and professionally supported programs, EM-related image-processing software is still at an earlier stage of development, and research in this domain is actively pursued in many laboratories. Nevertheless, the pro-

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grams already available—in many cases at little or no expense—amount to an extensive body of well-tested and user-friendly software, capable of a wide range of operations (Carragher and Smith, 1996).

IN THE EM, PROTEINS ARE VISUALIZED ONE MOLECULE AT A TIME

The primary attribute of the EM as an analytical tool is that its images relate to individual molecules, not vast ensembles. This is the case whether the molecules are ordered as in X-ray crystallography or dispersed as in other biophysical techniques such as ultracentrifugation or NMR spectroscopy. The physical basis of this capability is the charge of the electron, which makes it possible to accelerate electrons from an emitter, collimate them, and focus the resulting beam through electromagnetic lenses (Misell, 1978; Spence, 1980). A second important consequence of the electron's charge is that the beam interacts with the specimen strongly enough for a single protein molecule to yield a discernible, albeit noisy, image. However, the strength of this interaction is a two-edged sword.

To avoid degradation of the coherence and monochromaticity of the electron beam—and hence of its imaging qualities—by interactions with gas molecules, the EM column must be maintained under a high vacuum. Until recently, this vacuum has required that specimens be dehydrated before entering the EM column. Preservation of native protein conformations despite complete dehydration poses a severe, possibly unattainable, technical challenge. To date this problem has not been solved, and the various procedures whereby dehydration is accomplished in practice—which are useful and productive, at least to limited resolution—represent damage-control measures. However, with the recent innovation of cryo-EM (see discussion of Frozen-Hydrated Specimens), specimens remain fully hydrated, immobilized in vitreous ice (Stewart and Vigers, 1986; Dubochet et al., 1988; Booy, 1993; Chiu, 1993). Accordingly, this approach has unique potential for visualizing native protein structures in that it circumvents the fundamental problem of dehydration-induced denaturation.

Another obstacle posed by the electron charge, particularly for studies that aspire to higher resolutions, is radiation damage. The energy imparted to a protein molecule in a single inelastic scattering event is sufficient to rupture many bonds (Zeitler, 1990), so molecular structures degrade rapidly during observation. However, some workable solutions have been devised. For specimens prepared by negative staining or metal shadowing (see discussion of Specimen Preparation), the electrons that dominate the image are scattered not from the protein molecules themselves but from metallic replicas. Although not immune to the effects of electron irradiation, such replicas are much more resistant than unstained proteins. Nevertheless, even with unstained specimens, the adverse effects of electron irradiation may be minimized by employing imaging strategies in which the electron dose is minimized—i.e., virtually all of the electrons that pass through the protein are used to form its image (Williams and Fisher, 1970; Unwin and Henderson, 1975). Typically, a promising area of the specimen is identified at very low magnification (e.g., 2000×) at a negligibly low electron dose. Next, focusing, stigmation, and other initial adjustments are performed on a neighboring area. Finally the beam is switched on to the targeted area just long enough to record the micrograph. Hence, the observer shoots blindly, but not randomly. It also helps to ensure that (nearly) every electron counts by developing the negatives under conditions that maximize the detection efficiency of the film, or by using sensitive digital recorders—e.g., a charge-coupled device (CCD) camera (Brink and Chiu, 1994).

By operating the microscope at relatively low magnifications (30,000× to 50,000×), the total electron dose needed to achieve the required minimum darkening of the negative is correspondingly reduced (by the inverse-square law). However, it becomes counterproductive to reduce the magnification much further because of the limit set by the finite grain size of the recording medium (Downing and Grano, 1982), and because the lenses tend to work less well at lower magnifications—i.e., they are more susceptible to external perturbations.

THE NICHE OF EM IN PROTEIN STRUCTURE DETERMINATION

In general, the larger and more asymmetric a complex, the more difficult it is to solve its structure by X-ray crystallography. NMR spectroscopy, also capable of high resolution, is restricted in applicability to proteins up to about 25 to 30 kDa. In the past few years, EM has graduated into the elite class of experimental techniques that yield atomic models of proteins (see discussion of High-Resolution Studies).

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To date, the specimens that have been imaged in such detail have been highly ordered crystalline monolayers of intrinsic membrane proteins embedded in lipid bilayers, and, consequently, in their natural environment. However, even at lower resolution, many questions concerning the structures and interactions of proteins may be addressed readily and rewardingly by EM, and less easily if at all by other techniques. Some of these applications are outlined below: in several cases, a more detailed account follows later in the unit.

Assessing Purity and Determining Stoichiometry

EM provides a rapid and informative assay with respect to the purity and monodispersity of a preparation, particularly if the protein in question has a high molecular weight and/or is oligomeric. It is also economical: 10 ng of protein in a 2-μl droplet of buffer may suffice for an observation by negative staining (see discussion of Negative Staining). Electron micrographs are also a direct source of information concerning the shape, dimensions, symmetry, and architecture of protein molecules. The rotational symmetry and thence the stoichiometry of ring-like oligomers may often be determined by simple appraisal of micrographs. In ambiguous cases—e.g., "donuts" (see Fig. 17.2.1A and C)—the order of symmetry may be determined with greater confidence and objectivity by applying symmetry-detection algorithms (Crowther and Amos, 1971; Kocsis et al., 1995) or classification procedures (Dube et al., 1993) to sets of digitized images. If preliminary observations prove promising, the specimen may be suitable for a systematic analysis of its three-dimensional structure (see discussion of Architecture and Interactions of Protein Complexes). Moreover, if the shapes of two or more interacting components have been determined individually, EM of the complex may serve to define their docking geometry (see Fig. 17.2.1C).

Molecular-Weight Measurements of Individual Proteins

Scanning transmission electron microscopy (STEM) of unstained proteins provides a direct and unambiguous method of determining their molecular weights (Wall and Hainfeld, 1986; Müller et al., 1992; Thomas et al., 1994). This method is based on the principle that the number of electrons scattered by a molecule should be directly proportional to its molecular weight. In STEM imaging, this condition is met. The electron beam is focused down to a tiny probe only a few angstrom units (Å) across, to scan across the specimen. For each point sampled by this probe, the scattered electrons are collected in an annular detector situated behind the specimen and recorded as a digital signal. This imaging system provides an efficient dark-field mode (most electrons scattered are recorded), whose signal is linear over a wide range. As such, it is ideally suited for molecular-mass measurements.

These measurements are effected by integrating the image density associated with an object of interest in a digital micrograph, subject to an appropriate background subtraction to discount scattering from the support film. For this paradigm to hold, the protein should be unstained; also, the imaging mode should be linear (as it is, to a good approximation, in dark-field). Although these conditions cease to hold for very thick specimens (e.g., >500 Å) or when comparing molecules of quite different atomic compositions, the method works remarkably well for most proteins.

Structural preservation of the unstained molecules is enhanced by freeze-drying (Wall, 1979). Such preservation is not an absolute requirement for mass measurement of the whole molecule, but it may allow multiple domains to be visualized and consequently their masses to be determined on an individual basis (e.g., Cerritelli et al., 1996; Fig. 17.2.2).

To date, most EM-based mass analyses have been performed in the few laboratories that are equipped with a dedicated STEM, as distinct from a conventional TEM (transmission electron microscope) adapted with a STEM imaging mode. Notwithstanding their small number, the few biological STEMs in operation have been exceptionally productive. In particular, the STEMs operated at Brookhaven National Laboratory are accessible to user-defined projects (see discussion of Resources and User Facilities). Recent developments in energy-filtering electron microscopy promise to make EM-based molecular mass determinations more widely available in individual laboratories (Feja et al., 1997).

Although the remarkable progress of mass spectrometry has provided another analytical tool capable of operating in the elusive-high-molecular-weight range (see Chapter 16 for mass spectrometry techniques useful in this range), STEM retains some unique capabilities in this domain. For example, it is particularly well suited for large, asymmetric molecules; for the analysis of preparations that are het-

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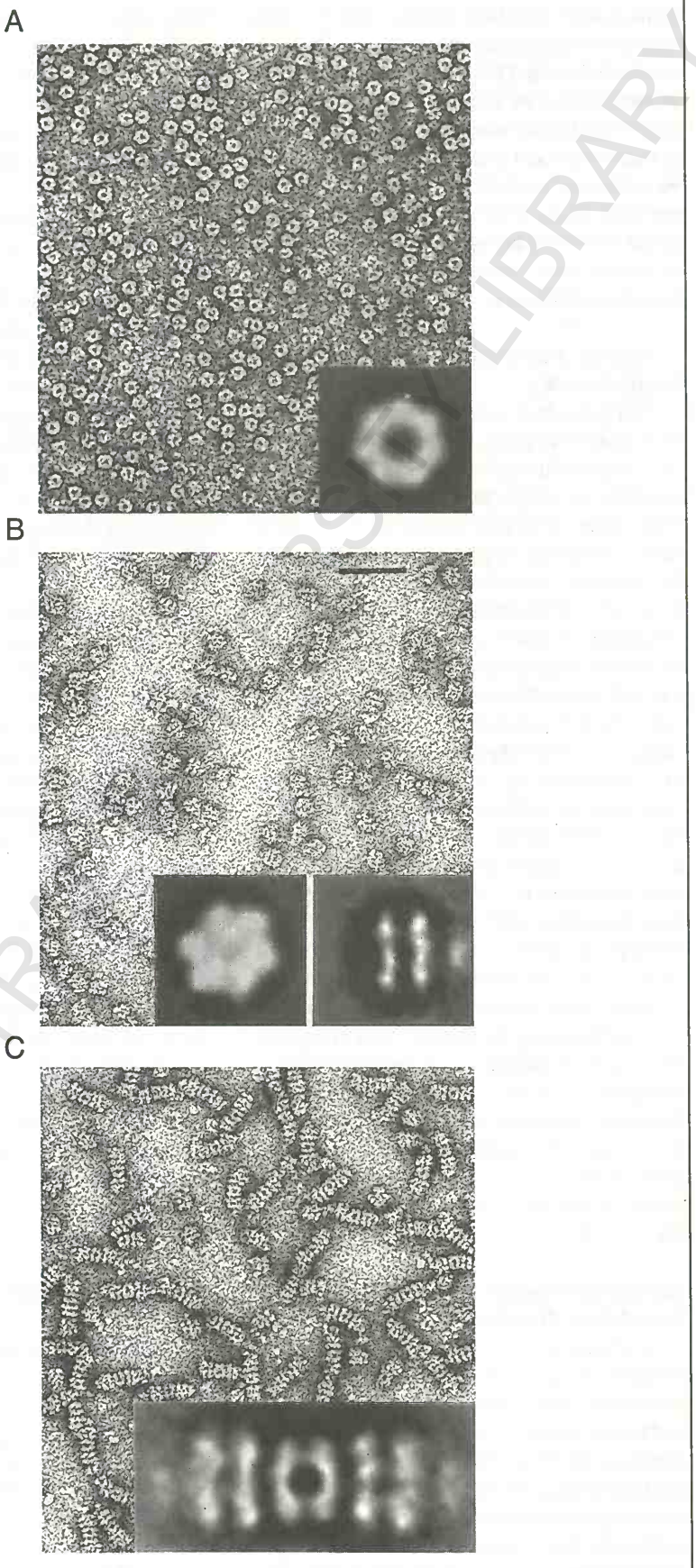

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erodisperse but in which the molecules of interest are recognizable; and for complexes such as nucleoproteins in which spatial information pertaining to the positions of protein-binding sites relative to the ends of a nucleic acid molecule provides a valuable complement to mass data (Thomas et al., 1994).

Architecture and Interactions of Protein Complexes

Higher-order structures constitute the class of proteins for which computer-enhanced EM holds the greatest potential. These complexes are typically composed of many subunits, and their masses range from several hundreds kilodaltons to many megadaltons. Their subunits may be arranged on a symmetric lattice—as in a viral capsid (see Fig. 17.2.3)—or they may be without symmetry, as in a ribosome. In either case, image averaging, applied in one form or another (Steven et al., 1991), is the foundation on which detailed structural analysis is based. Initially, such averaging was effected by Fourier filtration of regular two-dimensional arrays and thus was applicable only to this small class of specimens. Since then, the methodology has gradually generalized and diversified to the point where it may now be applied to virtually any kind of protein molecule, whether freestanding or polymerized (Frank, 1996; also see Fig. 17.2.4 for an illustrative analysis of freestanding molecules).

Although a detailed account of this methodology goes beyond the scope of this article, the principles are easily outlined. Analysis starts with collection of a suitably large set of molecular images (two-dimensional projections) of the highest possible quality. The required size of this data set depends on the dimensions of the molecule in question, the resolution aspired to, and whether analysis in two or three dimensions is envisaged. Typically, the data set ranges from a hundred or so to several thousand images (Henderson, 1995). For a two-dimensional analysis, the images are mutually aligned and sorted into intrinsically similar classes, and these are independently averaged. Structural conclusions are then drawn from these averaged images (see, e.g., Fig. 17.2.1).

For a full three-dimensional analysis, it is necessary to determine which view of the molecule is given by each image before a density map can be calculated. There are several strategies that may be followed. If a plausible, even if crude, three-dimensional model is available a priori, it may be projected computationally in all possible directions to generate a set of reference images that may be used to sort the data in a "supervised classification" (Penczek et al., 1994; Baker and Cheng, 1996). A more detailed model is then calculated from the sorted data, and the process iterated until no further improvement is obtained. Clearly, the end product of such a calculation has to be insensitive to the details of the starting model if it is to truly reflect the information content of the data and not just replicate the initial assumptions. If an appropriate starting model is not available, several strategies may be pursued to obtain one. For instance, internal symmetries may be exploited (Crowther, 1971) to determine the orientations of enough particles to calculate a first-generation model. More generally, the mutual orientations of averaged two-dimensional projections may be estimated by matching their common one-dimensional projections (a "common-lines" approach to "angular reconstitution"; van Heel, 1987). Alternatively, data from tilting experiments may be used to obtain a model—from either tilt pairs, as in the "random conical tilt" approach (Radermacher, 1988), or a full tilt series in the case of tomographic reconstruction (Dierksen et al., 1995).

Figure 17.2.1 *(at left)* Components of the energy-dependent protease, ClpAP, visualized in negative stain. The bar at the top of panel B is equal to 500 Å. Each panel shows the molecule as originally imaged (large field) and as enhanced by averaging, at higher magnification (inserts in bottom right of each panel). **(A)** ClpP (21-kDa monomer), the protease, is a ring-shaped molecule, ~115 Å in diameter. ClpP orients preferentially on EM grids to present its axial view, whereby the 7-fold rotational symmetry is revealed by rotational-symmetry analysis. ClpP consists of two 7-fold rings stacked to form a round, hollow complex that looks annular in projection from any direction. **(B)** ClpA (81-kDa monomer), the ATP-hydrolyzing, chaperonin-like component, forms hexamers in the presence of nucleotide which present two preferred views. The rarer top view discloses ClpA's 6-fold symmetry. The more prevalent side view presents two parallel striations, corresponding to the two tiers of domains (the subunit consists of two ATP-binding domains of approximately equal size). **(C)** Functional complexes of ClpP with ClpA are bipolar. They contain two molecules of ClpA, seen here in side view, one on either side of a ClpP molecule, also in side view. Adapted from Kessel et al. (1995) courtesy of Dr. M. Kessel.

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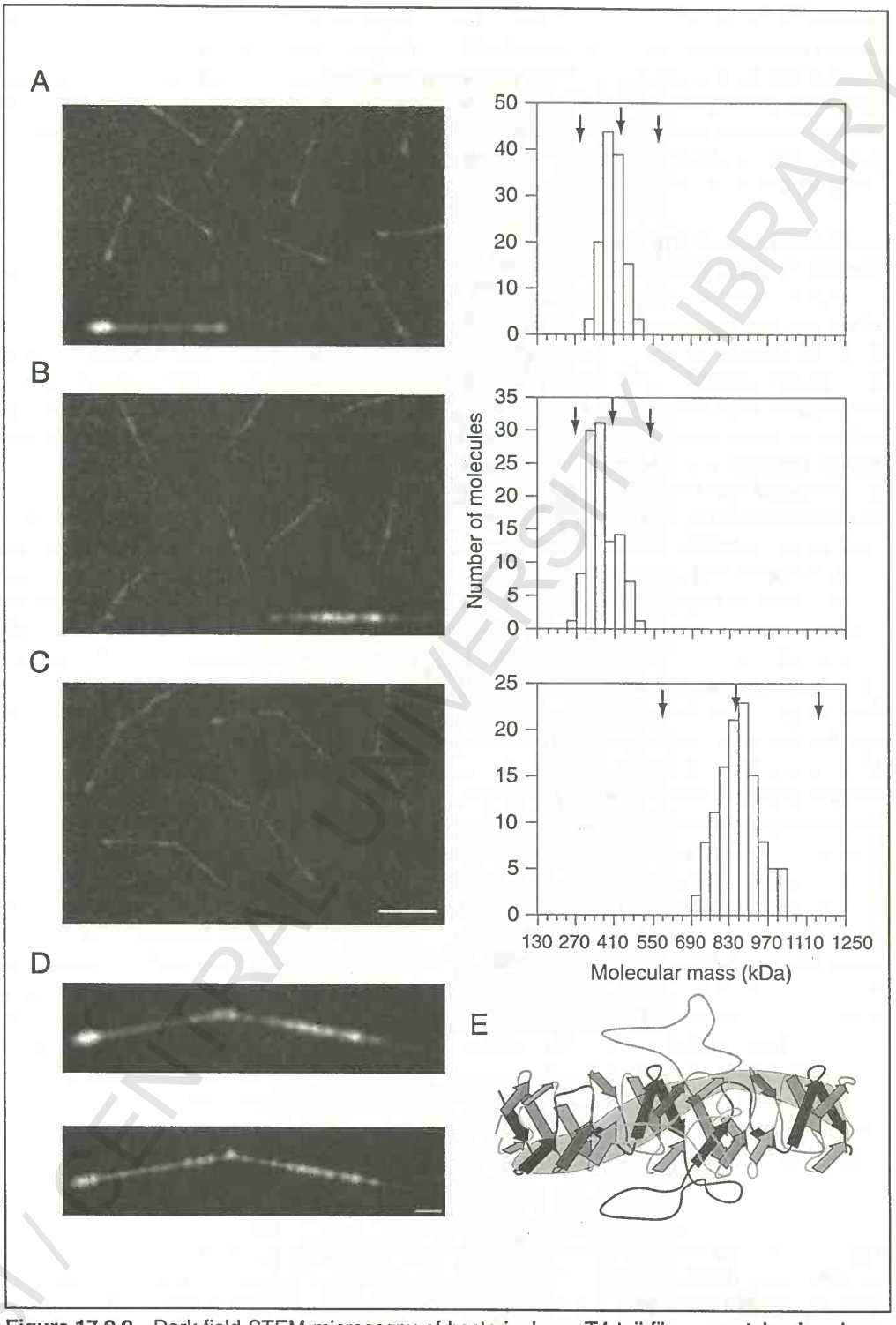

Figure 17.2.2 Dark-field STEM microscopy of bacteriophage T4 tail fibers, unstained and prepared by freeze-drying. Panels A, B, and C show micrographs alongside histograms of mass measurements made from data; the bar at the bottom of panel C is equal to 500 Å. **(A)** Proximal half-fibers; **(B)** Distal half-fibers; **(C)** Whole long tail-fibers. **(D)** Averaged images of the long tail fiber give consistent accounts of its domainal structure: top, unstained, freeze-dried; bottom, negatively stained with vanadate. **(E)** Molecular model of a short segment of the tail-fiber, as a three-stranded β helix. Adapted from Cerritelli et al. (1996) courtesy of Dr. M.E. Cerritelli.

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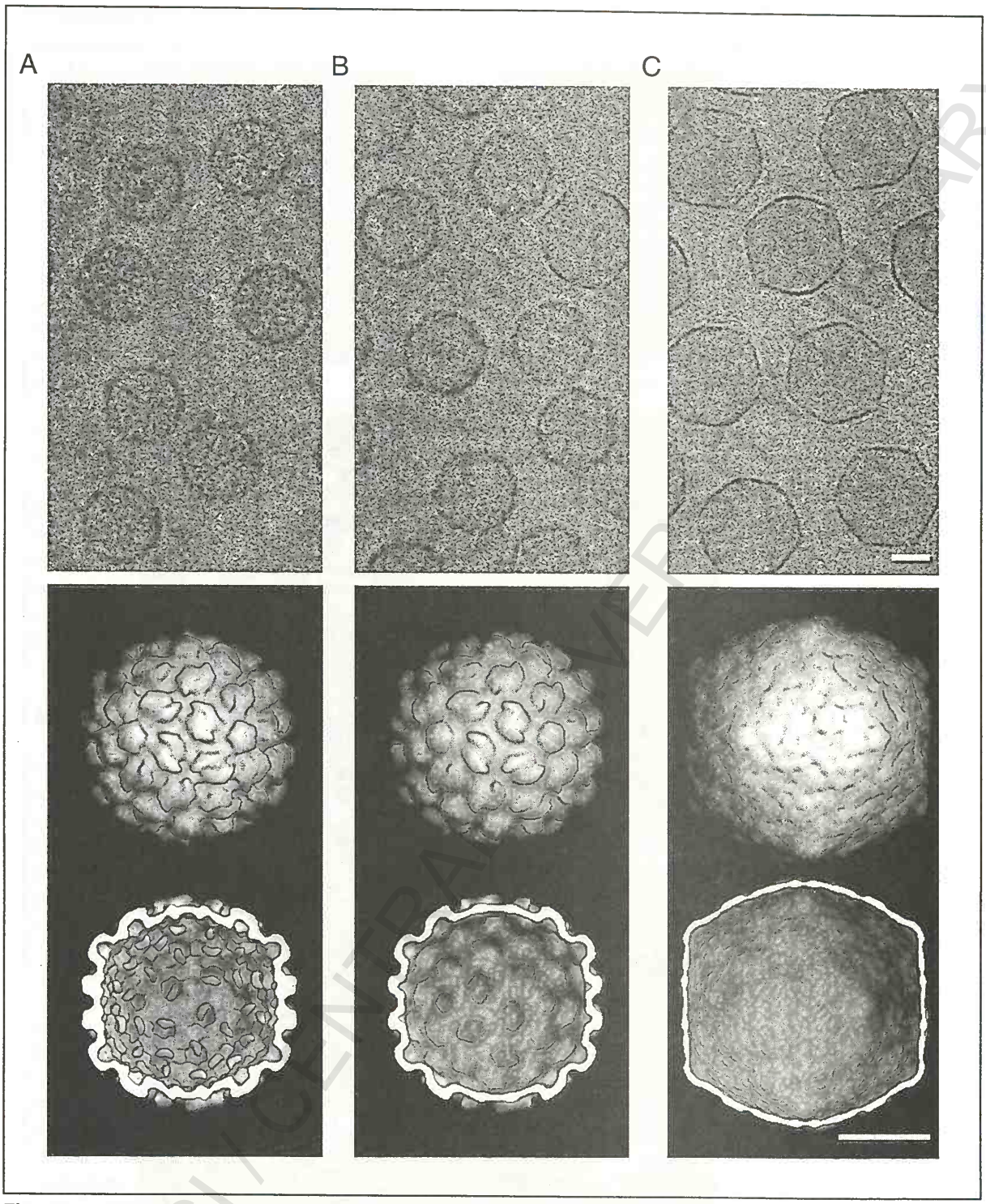

Figure 17.2.3 Cryo–electron micrographs of capsids of bacteriophage HK97. Original micrographs are shown in the top row of each panel (with the bar in this section of panel C equal to 200 Å on the scale of those micrographs); the outside surfaces of reconstructions at ~25-Å resolution are shown in the middle row; and their inner surfaces in the bottom row (with the bar in the bottom row of panel C equal to 200 Å on the scale of the reconstructions). (**A**) Prohead I, the earliest precursor, is composed of 420 copies of the 42-kDa capsid protein. (**B**) Prohead II, produced by proteolytic removal of 101 residues from the N terminus, which resides on the inner surface of Prohead I. (**C**) Head II is the end product of a large-scale conformational change in Prohead II that produces Head I, which then becomes covalently cross-linked together to yield Head II. Adapted from Conway et al. (1995) courtesy of Dr. J.F. Conway.

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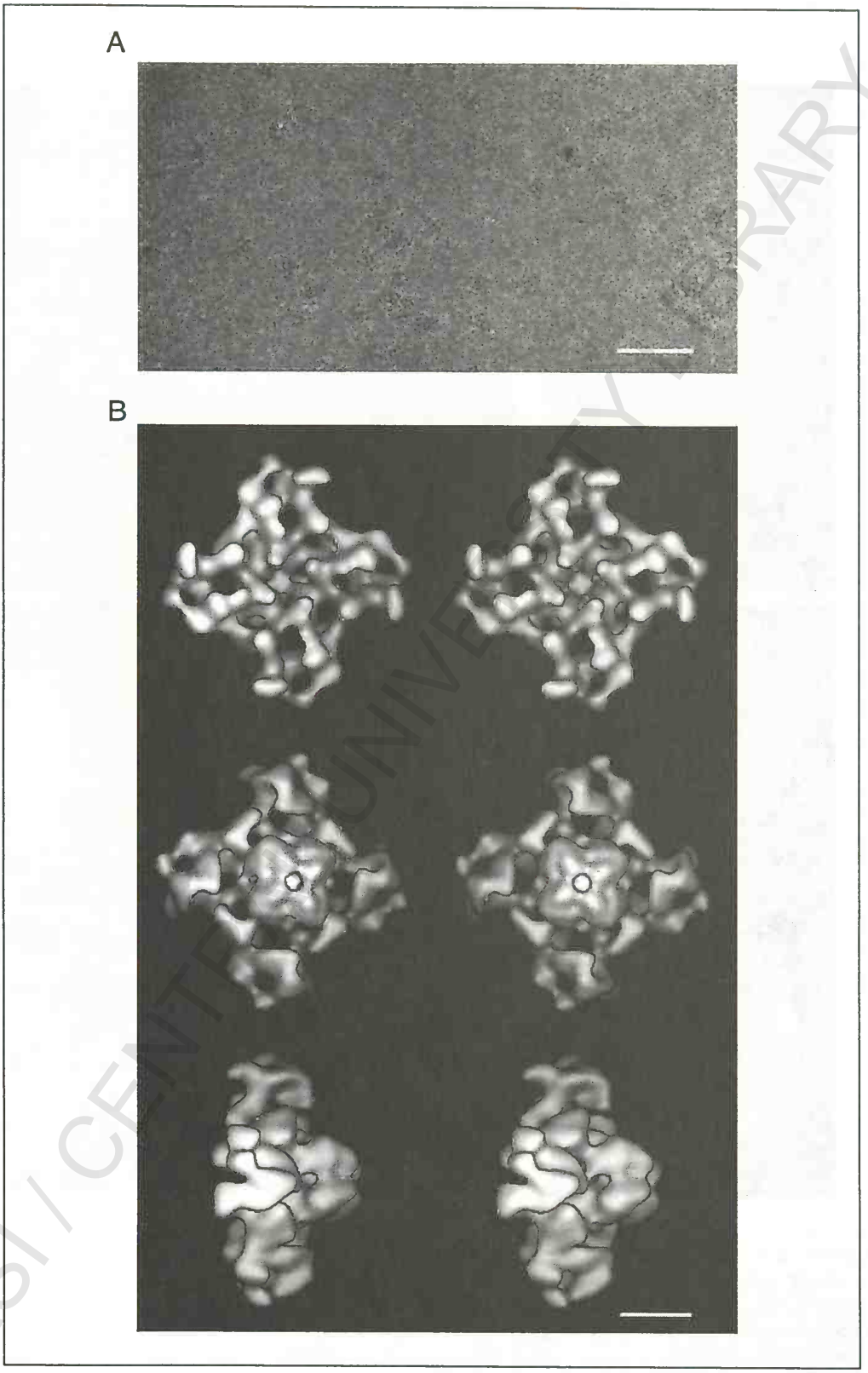

Figure 17.2.4 Electron micrography and reconstructions of calcium release channel. (**A**) Cryo–electron micrograph of calcium release channel. The bar is equal to 1000 Å. (**B**) Stereo pairs of a three-dimensional model of this tetrameric molecule (monomer mass = 170 kDa), as reconstructed to 25-Å resolution from cryo–electron micrographs by the random conical tilt method (Radermacher et al., 1994). The molecule is portrayed by surface rendering, as viewed along three mutually orthogonal axes. The bar is equal to 100 Å. Adapted from Radermacher et al. (1994) courtesy of Dr. M. Radermacher.

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As noted above, a rich variety of reconstruction strategies has been developed. However, one key condition applies: the raw images must contain enough information to permit reliable determination of the view to which each micrograph corresponds. Tomography is an exception to this rule because the relative orientations of the various tilts are specified by the observer. Thus, the larger and more asymmetric a complex, the more marked is the differentiation between its various views, and the more precisely one may hope to distinguish among them. The other side of this coin is that if the specimen size is doubled, one must then halve the uncertainty in its orientation angles to achieve the same resolution. In this context, small globular proteins will look essentially the same from any direction, even with micrographs of the highest possible quality, and thus will remain difficult if not impossible to tackle by single-particle methods. Nevertheless, such molecules are quite amenable to study by EM if organized into regular arrays (two-dimensional crystals or helices) in which the subunits are held in well-defined orientations (Jap et al., 1992).

Membrane Proteins

To facilitate the handling of intrinsic membrane proteins, such molecules are often solubilized in detergents, or else studied in genetically truncated form with their major hydrophobic elements excised or modified. For expediency, some investigations focus on expressing single domains or even peptide fragments for study, a strategy that is by no means confined to membrane proteins. While such provisions may provide material that is more amenable to in vitro biochemistry, it is worth bearing in mind that these fragments may no longer be membrane proteins and that isolated fragments may fail to adopt the conformations that they assume in the intact protein. Moreover, detergent-solubilized proteins may be significantly altered in structure by binding the surfactant. In this context the opportunity to visualize the intact proteins, even at limited resolution, is of considerable value. For instance, cryo-EM of lipoprotein vesicles and of natural or reconstituted membranes provides unique opportunities to visualize membrane proteins in their lipid-embedded environment (Brisson and Unwin, 1984).

Protein Filaments

Proteins that polymerize into helices are readily studied by EM. Although the first three-dimensional studies of helical polymers were performed almost 30 years ago (DeRosier and Klug, 1968), the methodology has continued to advance in applicability and resolving power (Stewart, 1988). Proteins that have been studied by this approach include cytoskeletal filaments as well as motility elements—either intracellular (actomyosin or microtubule-kinesin) or extracellular (e.g., flagellae; see discussion of Attainable Goals, below). Other examples include microbial adhesins (e.g., fimbriae; Heck et al., 1996) and nucleic acid–binding proteins such as the genetic recombination factor, recA (Stasiak and Egelman, 1994). In addition to naturally occurring and functionally active filaments, there are many cases on record of proteins polymerizing adventitiously into ordered tubes or filaments in vitro. Examples of proteins that have exhibited such behavior include the acetylcholine receptor (Brisson and Unwin, 1984) and the rev transactivator protein of HIV (Wingfield et al., 1991). In such cases, the ordered packing of molecules may be of dubious biological significance, but their helical symmetry is convenient for structural analysis in that they hold the subunits in well-defined orientations—helical filaments are one-dimensional crystals.

A tentative generalization concerning proteins that have a tendency or a functional requirement to polymerize into filaments is that they are refractory to forming well-ordered three-dimensional crystals. Under conditions that cause such proteins to precipitate, the interactions that lead to filament formation may be expected to assert themselves. Thus, filament formation is likely to preempt three-dimensional crystallization, except in the rare cases in which the helical symmetry allows filaments to pack together to generate a three-dimensional crystal of some repeating unit. More generally, disordered lateral packing of filaments into tactoids or paracrystals will occur. This obstacle to crystal formation is circumvented in the case of filament-forming proteins that bind a ligand masking their self-interacting surface, thus providing a binary complex that may be amenable to co-crystallization. In the event that a high-resolution structure of the subunit can be obtained, either directly (e.g., for the gonococcal pilin; Parge et al., 1995) or from cocrystals (e.g., of actin complexed with DNase I; Kabsch et al., 1990), three-dimensional EM may be a source of information on the intersubunit interactions that govern their packing in the filament (Holmes et al., 1993; Aebi et al., 1996).

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Fibrous Oligomers

Many proteins consist of one or more high-molecular-weight subunits that have extended, often flexible, conformations. Such molecules are particularly prevalent in "connectivity" interactions in such settings as the extracellular matrix, receptor recognition, and cytoskeleton-associated phenomena. To a first approximation, they may be considered as one-dimensional proteins, since their folds often employ one or more of the standard fibrous-protein motifs—coiled-coil α helices or collagen-like 3_{10} helices (Fraser and MacRae, 1973) or the recently discovered β helix (Yoder et al., 1993)—or else they consist of multiple globular domains connected by fibrous linkers. The former two motifs may be readily detected by analyzing the protein's amino acid sequence—i.e., coiled-coil α helices consist of multiple tandem copies of canonical heptads (Cohen and Parry, 1990) and collagen-like sequences tend to have glycine in every third position. Algorithms have been developed to detect the presence of heptads in a sequence (Lupas et al., 1991) and reasonable predictions may be made with respect to the packing of the α helices in coiled coils (i.e., the number of strands and their mutual orientation) on the basis of empirical rules concerning the occupancy of key sites in the heptads (Woolfson and Alber, 1995).

For oligomeric fibrous proteins, EM can serve as an important source of structural information that complements sequence-based predictions. The first order of business is to acquire an image that is sufficiently detailed to divulge the molecule's domainal structure, which may then be correlated with inferences based on the primary sequence, as noted above. The information required for such a correlation—i.e., the number of distinct domains, their lengths and widths (in the case of rod domains), and their sizes and spacing (for globular domains)—may be inferred from averaged electron micrographs in the moderate-resolution range of 20 to 30 Å. For such analyses, fibrous proteins have the advantage that they are almost always present in a definite view—the side view. Direct imaging can be very effective with such proteins (e.g., myosin; Walker et al., 1985) and image averaging adds a valuable refinement. Intrinsic flexibility may result in variations from particle to particle that pose a problem for averaging, but this may be rectified by computational straightening (Egelman, 1986; Steven et al., 1991).

By combining EM with sequence-based predictions as outlined above, it may be possible to develop a model for the overall path of the polypeptide chains through the molecule. A number of fibrous proteins have been analyzed in this way, including the tail fibers of bacteriophages T7 (Steven et al., 1988) and T4 (Cerritelli et al., 1996; see Fig. 17.2.2) and the filamentous hemagglutinin of *Bordetella pertussis* (Makhov et al., 1994). This form of analysis appears to have considerable potential for the study of other fibrous proteins.

An alternative approach to the study of large filamentous proteins is via high-resolution studies of domains by crystallography (Strelkov et al., 1996) or NMR spectroscopy (Baron et al., 1991; Pascual et al., 1996). As a prelude to such studies, the domain of interest must be identified, expressed in bulk, and purified. The decision as to what to express is, of course, pivotal; the number of candidates is large and the number for which in-depth study is practical is small. EM has the potential to reveal the full domainal organization of such proteins. EM may also help clarify whether an expressed domain of a fibrous protein has folded correctly.

Visualizing Conformational Changes and Switches

Conformational changes control the assembly and activation of many protein complexes. The scale of these structural revisions varies widely. At one extreme, subtle local changes may suffice for a substantial alteration in the affinity of a binding site. At the other extreme, movements of structural elements over distances of 25 Å or more (Jontes et al., 1995) and extensive refolding of polypeptide chains (e.g., in bacteriophage T4 capsid maturation; Black et al., 1994) accompany some of the more dramatic conformational changes on record. Other examples of this phenomenon include activation of reovirus to a transcriptionally competent state (Dryden et al., 1993), herpesvirus capsid maturation (Trus et al., 1996), and chaperonin switching upon binding nucleotides (Roseman et al., 1996). EM provides a powerful tool to study conformational transitions, particularly those in which large-scale structural changes take place (see Fig. 17.2.3).

The suitability of EM for this application is enhanced by the consideration that differential changes may be detected with considerably greater sensitivity than the nominal resolution of the analysis implies (see discussion of Immuno-EM). A further consideration is that the distinct states in a functional cycle or sequence may vary considerably in stability, with some

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states being relatively short-lived. Instability is not conducive to the success of crystallography in that the proteins may well switch into a lower-energy state prior to or during crystallization. However, EM requires only that a conformation be sustained long enough to allow observation. By judicious use of rapid freezing techniques to capture short-lived states, the time resolution achievable in cryo-EM studies is now of the order of a few milliseconds (Berriman and Unwin, 1994; Walker et al., 1995).

CONCEPTS AND MISCONCEPTIONS

To some, electron microscopy has the dubious reputation of having little to offer for the study of protein structure. According to this point of view, first of all, it is prone to artifacts—i.e., "you can see anything you want." Second, too small a fraction of molecules in a bulk population are observed for the results to be statistically meaningful. Third, the resolution is too low to yield worthwhile information.

Artifacts and How to Avoid Them

In principle, artifacts can arise from two major sources. The first relates to failure of the observer to identify the molecule of interest correctly thus basing conclusions on some other component present on the grid. With the second, the molecule of interest is correctly identified, but the acts of specimen preparation and observation (e.g., radiation damage) have caused changes, random and/or systematic, in its native structure that are sufficiently large to seriously mislead the observer. In practice, the risk of both kinds of artifacts may be largely eliminated by observing a few straightforward precautions.

One essential measure is to avoid contamination of the EM grid. It should be standard operating procedure to use double-distilled water and chemicals of the highest available purity in making specimen buffers and grid-washing solutions. It should be recalled that sterility is not the same thing as cleanliness. The specimen substrate, usually a thin carbon film, should be clean; glow-discharging or casting the film immediately before use can be helpful provisions. The risk of cross-contamination from tweezers used to prepare grids of other specimens should be borne in mind. Cellulose fibers from filter paper used to blot grids should not be mistaken for protein filaments, and vice versa. One distinguishing criterion is that protein filaments often exhibit distinctive substructure such as striations or serrations in the size range typical of individual subunits or domains (20 to 50 Å) that are not seen on cellulose fibers.

The Cardinal Importance of Reproducibility

When examining a new protein by EM, the first priority is to establish conditions under which the appearance of the particles is fully reproducible. It is also desirable to optimize their distribution on the grid by systematically varying the sample concentration and other experimental conditions (such as grid-washing procedure). Optimal concentrations tend to vary from protein to protein, but typically, 10 to 50 µg/ml may be appropriate for specimens adsorbed to a carbon film for negative staining or nebulized onto a mica surface for shadowing; 0.5 to 2.5 mg/ml may be appropriate when preparing thin films of frozen suspensions for cryo-EM (see discussion of Specimen Preparation Techniques for more information on these procedures). Once an adequate distribution of particles has been achieved, it should be determined if they are uniform in size and appearance and if there are any indications of substructure. For example, featureless globular particles of randomly varying size seen in a negative-staining experiment should be viewed with suspicion. They may simply represent an emulsion of grease droplets or bubbles somehow formed in the stain layer. If the observed particles show distinctive substructure but fall into several size classes, there are several testable possibilities. Particles that are oligomeric in solution may be breaking down when prepared for EM. This hypothesis may be tested by cross-linking the preparation with glutaraldehyde, which should suppress this tendency to break down, or by repeating the procedure using cryo-EM, in which specimens are not subjected to such extreme forces.

Alternatively, the specimen may be impure or inhomogeneous. On occasion, protein preparations that seem to be quite pure according to SDS-PAGE (*UNITS 10.1 & 10.4*) may look considerably less pure by EM. For instance, there may be contaminants that contribute only faint gel bands, but cumulatively amount to a significant fraction of the total molecular population. Also, some of these intruders may have higher binding affinities for the support film than the majority protein of interest and thus be overrepresented in micrographs.

A further important reproducibility check, particularly if doubt persists, is consistency of

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appearance of the same specimens when prepared for EM in quite different ways (e.g., staining and shadowing or cryo-EM).

Finally, in identifying the molecule of interest in micrographs, it is important to take into consideration all other available information. If the protein has been purified by gel-filtration chromatography (*UNIT 8.3*) or sucrose-gradient centrifugation, an estimate of its molecular weight should be available. Within the limited scope of such a calculation, that molecular weight should be consistent with a particle of the size seen in the EM images. A typical value for the partial specific volume of protein should be compared with the estimated particle volume. Assuming that the particle is correctly identified, it should be determined whether the observed features make sense in the light of what else is known.

Variability in Appearance

If the particles seen in electron micrographs are approximately uniform in size but distinctively variable in appearance, several explanations are possible. It may be that the protein of interest assumes several different orientations on the EM grid, thus presenting distinct views. If that is so, this property may be exploited to yield a three-dimensional representation of the protein by synthesizing the various two-dimensional projections (Frank, 1996). One check is to perform tilting experiments: suitable rotation of the specimen in the EM may convert one view into another. Alternatively, the preparation may contain two or more similarly sized proteins. To evaluate this possibility, it is essential to make a careful review of the biochemical evidence pertaining to purity. In cases of ambiguity, specific antibodies to the protein of interest, if available, can be used to advantage. The question here is: do the antibodies label all particles or only a specific subset?

Measuring the Dimensions of Protein Molecules and Polymers

In principle, systematic errors of several kinds may affect size measurements made by EM. In practice, awareness of such effects makes it possible to minimize their impact. For instance, the magnification may diverge by up to 5% or so from its nominal value because of lens hysteresis and the state of alignment of the microscope column. This limitation may be overcome by employing an internal calibration—i.e., a specimen that is easily recognized and whose dimensions or spacings in solution are known, such as the plate-like crystals of catalase (Wrigley, 1968) or tobacco mosaic virus (Wall, 1979) with its distinctive 23-Å axial repeat. A second problem is that proteins may spread when they are dried down on a support film, thus exaggerating their size, or they may shrink upon drying and appear smaller. The advent of cryo-EM has greatly reduced uncertainties associated with the state of preservation of the specimen. Thus, it is usually possible to determine the dimensions of protein molecules to within a few percent for freestanding specimens; the main source of uncertainty arises from imprecise edge detection with images of limited resolution. With periodic specimens, edge detection is less of an issue because spacings may be determined from diffraction patterns to within ≤1%, and in favorable cases to even higher precision.

Reproducibility Again: Statistically Meaningful Results

This section concludes with a reiteration of the importance of reproducibility. The sample should be reproducible from one biochemical preparation to another and should yield consistent results in independent EM experiments. For studies involving the use of image processing, the reproducibility of the end-product images should be assessed in quantitative terms.

Finally, consider statistical representativity. Suppose that a sample consists of 0.1 ml of a solution containing a 250-kDa protein at 0.5 mg/ml—a total of ~0.3×10^{14} molecules. If an EM study is based on, say, 3000 mutually consistent images, these molecules amount only to an infinitesimal fraction (~10^{-10}) of the total population. Can they be taken as statistically representative? Consider the following proposition: suppose the population consists of two kinds of microscopic particles: the protein of interest (fraction p), and contaminants (fraction $1-p$). Then, in an unbiased sampling experiment of 3000 trials, the probability of picking out only contaminants is $(1-p)^{3000}$—an extremely small number for any reasonable value of p. Of course, this proposition is idealized, particularly with respect to the assumed lack of bias in the trial, which in practice may be affected by selective adsorption of contaminants to the EM grid and subjectivity on the part of the observer. It does, however, make a point with respect to the issue of sample sizes and statistical representativity.

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RESOLUTION

Instrumental Factors

The resolution achieved in an EM study of proteins is constrained by many factors. Among these, it is possible to distinguish between instrumental factors and specimen-dependent factors. In purely optical terms, the wavelength of the electrons is not a resolution-limiting factor, as it is in light optics. At the commonly used accelerating voltage of 80 kV, the wavelength of the electrons is ~0.04 Å. Nor, in principle, is the outcome of most experiments limited by the intrinsic resolving power of the microscope. Commercially available instruments routinely attain resolutions of 2.5 to 3.0 Å when correctly aligned and operated close to focus and with the lenses fully excited—i.e., at very high magnification (for example, 400,000×)—for imaging of radiation-resistant inorganic specimens. Structural information at this resolution should allow the tracing of polypeptide chains.

Why is this resolving power not generally realized with biological material? The answer is, at best, complex and incomplete. Many factors are involved. One major consideration is the need to minimize radiation damage when imaging biological macromolecules. In consequence, the EM is operated at lower magnifications, where it tends to perform less well—e.g., at these magnifications EM performance is more susceptible to impairment by stray magnetic fields. Another consideration relates to the size of proteins and the complexity of their substructure, and the large numbers of precisely defined views that are needed to determine their three-dimensional structures to high resolution (see discussion of Quantitative Resolution Criteria in Two and Three Dimensions).

The instrumental resolution limit of a given micrograph may be assessed from its diffraction pattern (obtained optically or by digital Fourier transform; see Fig. 17.2.5C). The spacing of the outer limit of intensity in this pattern represents the maximum resolution that the image is potentially capable of yielding.

Specimen-Dependent Factors

Because of specimen-related factors, the resolution limit of biologically meaningful information is usually considerably lower than the instrumentation-imposed limit. One such specimen-related factor is the possibility of random distortions in native structure induced by specimen preparation. Another is radiation damage. "Shot noise" in images that were recorded at very low dose to reduce radiation damage and in consequence, are statistically ill-defined, as well as metal-grain size (if shadowing or staining are used) are additional specimen-related constraints on the resolution limit. Since resolution may be constrained by so many factors, any of which may be limiting in a given case, a pragmatic measure is to define the **effective** resolution of an EM experiment in terms of the limit to which structure is consistently represented over a substantial population of molecules (see Quantitative Resolution in Two and Three Dimensions).

Resolution and Signal-to-Noise Ratio

As noted above, electron micrographs of proteins are noisy images. The sources of noise are many (Frank, 1996), and a full accounting will not be attempted here. In general, however, the finer a detail, the more likely it is to be obscured by noise. This problem may be alleviated by image averaging. In its simplest form, this involves visual appraisal of an array of like images and identifying common features. On a more systematic basis, averaging is performed by digital summation of many individual images, appropriately aligned. Each image is assumed to consist of a signal overlaid with additive noise; if it is random, the noise is suppressed by averaging; if it is consistent, the signal is reinforced. In this context, the resolution criteria outlined below apply only to averaged images. The same information is, of course, latently present in individual images; the problem is that it cannot be inferred unambiguously without image averaging.

Quantitative Resolution Criteria in Two and Three Dimensions

For periodic specimens—e.g., crystals or helices—the resolution limit may readily be calculated in terms of the spatial frequency of the outermost periodic reflections that are visible above background in the diffraction patterns (e.g., Fig. 17.2.5B). For EM data (images, not electron-diffraction patterns) this limit is a conservative one and may be an underestimate because of lattice disorders. The adverse effects of disorder, which result in smearing of the averaged image with a consequent reduction in resolution, may be mitigated by computational correction of the lattice (van Heel and Frank, 1981; Steven et al., 1991). This procedure may yield a significant improvement in the resolution of the averaged image (see Fig. 17.2.5).

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For nonperiodic specimens, corresponding measures of resolution have been expressed in terms of mathematically defined criteria applicable to sets of digitized images (each representing a distinct copy of the same protein). Several such criteria are widely used—e.g., the differential phase residual (Frank et al., 1981), the Fourier ring correlation coefficient (Saxton and Baumeister, 1982), and the spectral signal-to-noise ratio (Unser et al., 1987). The numbers that these criteria give for the resolution of a given data set tend to vary slightly because different thresholds are used to define the limit of resolution. Although such variations are small, studies seeking incremental improvements in resolution on a given specimen should apply the same criterion consistently. Similarly, when comparing several studies of the same protein, investigators should consider whether any small differences in reported resolution may not simply reflect the choice of resolution criterion.

The resolution of two-dimensional images imposes an upper bound on the resolution of the three-dimensional density map that may be calculated from them. In three dimensions, however, an additional resolution-limiting factor comes into play—i.e., the question of how fully and how uniformly the underlying set of two-dimensional views covers three-dimen-

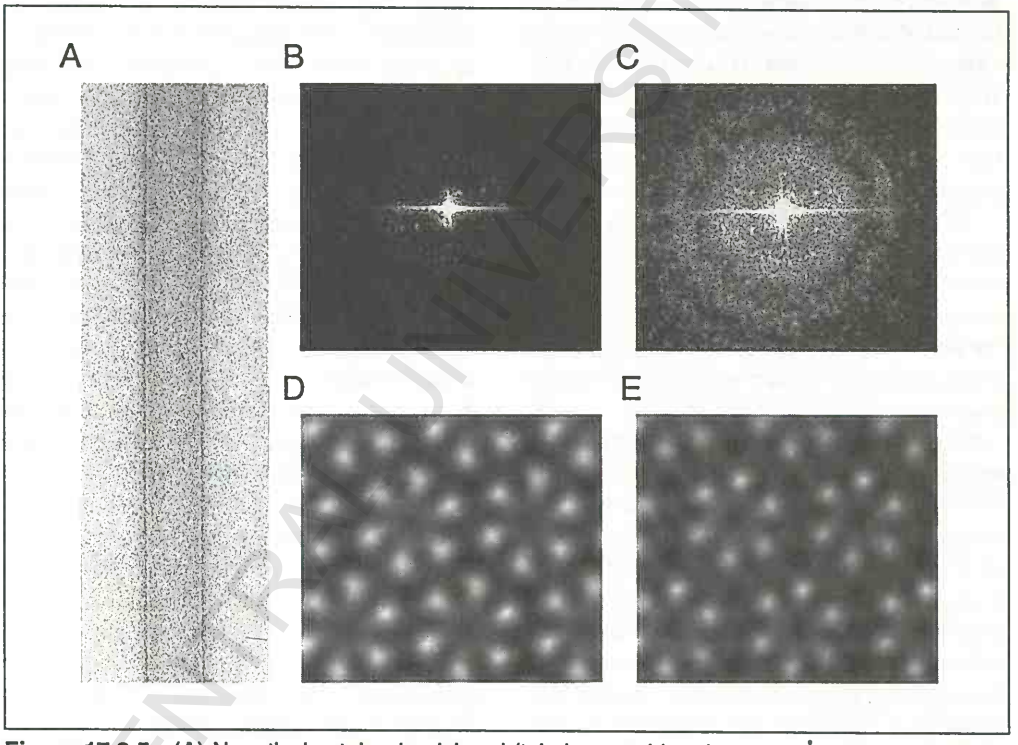

Figure 17.2.5 (**A**) Negatively stained polyhead (tubular capsid variant; 750-Å wide) of bacteriophage T4. (**B**) Optical diffraction pattern showing double hexagonal reciprocal lattice (from the top and bottom surfaces of the flattened tube). Periodic reflections extend to a resolution of 30 Å. (**C**) Optical diffraction pattern of same micrograph, overexposed to illustrate spectral limit of potentially accessible information (~12 Å). (**D,E**) Correlation averaged surface lattice images. The actual resolution achieved is 22 Å, indicating both that computational correction for lattice disorders has improved the resolution from 30 to 22 Å, and that the "signal" generated by the stain beyond that limit (i.e., between 22 Å and 12 Å) is not reproducible from capsomer to capsomer. The hexagonal surface lattices are shown for the "cleaved, expanded" state (panel E) and the "uncleaved, expanded" state (panel D). Upon maturation, the T4 surface lattice undergoes a major conformational change in which its lattice constant increases from 118 to 140 Å in the "expanded" state. Normally, this transition is preceded by proteolytic cleavage of the capsid protein, gp23, from its initial molecular weight of 56,000 Da to 49,000 Da, yielding the "cleaved, expanded" state (see Black et al., 1994). However, expansion may be induced in vitro in the absence of cleavage by treatment with low concentrations of guandine hydrochloride or urea, giving the "uncleaved, expanded" state. Comparison of these images allows the 7-kDa "Δ-domain" (which is removed by the cleavage reaction) to be localized: additional densities are visible around the rim of the proteomers of the uncleaved lattice (panel D). The center-to-center spacing between hexameters in the surface lattice is 140 Å. Adapted from Kocsis et al. (1997) courtesy of Dr. E. Kocsis.

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sional space. For a three-dimensional map calculated from N equally spaced projections of a particle of diameter D, an estimate of the resolution (r) is given by $r \cong \pi \times (D/N)$ (Klug and Crowther, 1972). With fewer views or irregularly spaced views, the resolution deteriorates. One specific case—anisotropic resolution—encountered because of the limited angular range accessible in a tilting experiment, is considered later in the unit (see discussion of Mapping Specific Components in Large Protein Complexes).

Attainable Goals: The Art of the Possible

The following discussion briefly summarizes goals that are realistically achievable with current methodology for several classes of protein specimens.

Two-dimensional crystals

With highly ordered molecular monolayers, electron crystallography can achieve resolutions of ~3.0 Å in the plane (see discussion of High-Resolution Studies). With such three-dimensional density maps, it is possible to trace polypeptide chains and render a detailed account of the molecular conformation, including the locations and dispositions of prosthetic groups (Kühlbrandt et al., 1994). For less-well-ordered two-dimensional crystals embedded in ice or sucrose, a more realistic goal may be a density map at 6- to 8-Å resolution. This is sufficient to describe the configuration of a bundle of α helices, a capability that is valuable for the study of intrinsic membrane proteins, many of which have transmembrane segments that are α-helical. As a prelude to detailed analysis of unstained specimens, two-dimensional and three-dimensional analyses of stained or shadowed specimens in the resolution range of 15 to 30 Å are eminently worthwhile.

In general, surface details (e.g., loops connecting α helices) are visualized less well in such maps, even at high resolution, because of the limited tilt range covered (see discussion of High-Resolution Studies) and possibly also because of interactions with the support film or air interface. To overcome this limitation, at least in part, high-resolution shadowing (Fuchs et al., 1995) or atomic force microscopy (Shao and Yang, 1995; Engel and Gaub, 1997) offer important sources of complementary information on surface detail.

Helical polymers

The three-dimensional structures of filaments may readily be calculated to resolutions of 20 to 30 Å for negatively stained or ice-embedded specimens. These resolutions are sufficient to define the shapes and packings of their subunits. The binding of ancillary proteins to filaments may be studied in detail by difference mapping. If an atomic model is available for the subunit, EM may be used to determine how they associate in the filament and to interpret induced structural changes (Stasiak and Egelman, 1994; Aebi et al., 1996). With bacterial flagellae, it has been possible to extend the resolution to ~10 Å by combining many images, thereby yielding information on the packing of α helices in the filament backbone (Mimori et al., 1995; Morgan et al., 1995). The highest resolution to date with helical specimens, ~8 Å, has been achieved with acetylcholine receptors packed in crystalline membrane tubes, again revealing the locations and dispositions of α helices (Unwin, 1996). As a class, filaments have some liabilities as compared with planar specimens, but they have the advantage of providing a full range of angular views (after combining multiple particles), so their density maps are not affected by anisotropic resolution.

Oligomeric fibrous proteins

When adsorbed to a thin carbon film for visualization by EM, fibrous oligomers usually present a side view. Their domainal structures may be inferred directly from micrographs, or more reliably, after image averaging. So far, negative staining has been the method of choice because of its high signal-to-noise ratio, which is necessary for precise computational straightening of these thin, curved molecules. Resolutions of 20 Å or so are usually attainable. If integrated with information from other sources—primarily sequence-based predictions, which are relatively reliable in the case of fibrous-protein motifs—it may be possible to sketch out the overall path followed by the polypeptide chain through the molecule (Steven et al., 1988; also see Fig. 17.2.2).

Icosahedral viruses

As a geometrically defined class of specimen, spherical viruses occupy a special niche. On one hand, they are single particles and imaged as such; on the other hand, they possess a high degree of internal symmetry that may be exploited for purposes of structural analysis. A

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formalism for calculating the three-dimensional structures of spherical (i.e., icosahedrally symmetric) virus capsids was developed long ago (Crowther, 1971). However, this approach came to fruition only after the advent of cryo-EM (Adrian et al., 1984; Fuller, 1987; Baker et al., 1988), whereupon it has been applied with great effect to many capsids and derivatives thereof (reviewed by Steven et al., 1997). The resolutions attained, initially in the range of ~35 Å to ~45 Å, have improved steadily. Recent developments have pushed the resolution down below 10 Å for favorable specimens, revealing elements of secondary structure (i.e., α helices; Conway et al., 1997; Trus et al., 1997).

Isolated protein complexes

There are also some large multienzyme complexes that conform to icosahedral symmetry, and accordingly may be analyzed by the same procedures as are used to study virus capsids (Stoops et al., 1992). However, protein or nucleoprotein complexes that observe lower symmetry or no symmetry constitute a much broader class of specimens, and several general approaches have been developed for calculating their three-dimensional structures from sets of images of freestanding particles (reviewed by Frank, 1996). Such complexes do not necessarily possess any symmetry, but if they do, it may be exploited. Most studies start with negative staining (see discussion of Specimen Preparation). Distinctive two-dimensional projections of the complex are recognized and averaged to yield images that are typically in the resolution range of 20 to 30 Å, and these may be synthesized into a three-dimensional density map. These experiments may then be followed up with cryo-EM, with its improved preservation and direct representation of native structure (as distinguished from the indirect representation afforded by a stain distribution). Currently, resolutions in the range of 20 to 25 Å are attained in such three-dimensional maps (Frank et al., 1995; Stark et al., 1995) and there is every reason to anticipate progress to higher resolutions in the near future.

SPECIMEN PREPARATION: YOU GET OUT WHAT YOU PUT IN

Negative Staining

The principle of negative staining (Hayat and Miller, 1990) is to enhance the low intrinsic contrast of a protein by embedding it in a dense medium (Fig. 17.2.6B). The protein molds the surrounding stain, and its shape is ultimately deduced from that of the stain distribution, which scatters most of the electrons. In principle, the mode of dehydration employed in negative staining—air drying—should be severely disruptive to native protein conformations. The high concentrations of heavy-metal salts and abrupt changes in pH imposed during staining are also causes for concern. Notwithstanding, several negative-staining studies of model proteins have documented excellent correspondence between experimental images and images calculated from the known, native, structure (Steven and Navia, 1980). Moreover, the technique has been found to work surprisingly well on a wide range of specimens (Bremer et al., 1992). To an increasing extent nowadays, negative staining serves as a prelude to more detailed investigation by cryo-EM.

The main advantages of negative staining are that it is quick and easy to perform and has a generally sound record as attested to by studies with model proteins and with specimens subsequently analyzed by cryo-EM. Ease of use and the capability for screening many samples rapidly and inexpensively are likely to guarantee the continuing popularity of this technique. Its limitations relate mainly to structural preservation, especially where fine details are concerned, and the fact that the stain depicts only surface features, yielding little information about internal structure other than the presence of stain-penetrable cavities (see Fig. 17.2.1A and C).

Shadowing

Shadowing experiments also use heavy metals to amplify the low intrinsic contrast of proteins. In this case, the metal is applied by evaporation to form a thin coating on predried specimens (Fig. 17.2.6C). Dehydration is usually effected by air drying, which is technically the simplest drying method but which usually provides poor preservation, or freeze-drying, which generally gives superior preservation (Kistler et al., 1977; Nermut, 1977; Heuser, 1983). Critical-point drying represents a third option (Newcomb and Brown, 1989). Nebulizing droplets of a protein-containing glycerol solution onto a mica surface prior to drying under vacuum at room temperature has been found to mitigate the adverse effects of this form of air drying, presumably because of the conformation-preserving effects of glycerol. This technique has been particularly efficacious as applied to filamentous proteins (Tyler

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and Branton, 1980; Milam and Erickson, 1982; Fig. 17.2.7).

The metals most commonly used for shadowing are platinum and tungsten/tantalum. Apart from the state of preservation of the specimen, the major resolution-limiting factor is the grain size of the deposited metal, which in the case of platinum depends on the temperature of the specimen during shadowing. Low temperatures (e.g., <–80°C) limit the surface mobility of incident metal atoms and are conducive to small grains. Room-temperature shadowing is accompanied by greater surface mobility of the atoms (larger, fewer grains). The latter property may be exploited to localize nucleation hot spots on proteins in a technique called "decoration" (Weinkauf et al., 1991).

Metal may be evaporated either unidirectionally onto a static specimen or by rotating the specimen while the shadowing is taking place (Fig. 17.2.6C). Unidirectionally shadowed images allow specimen heights to be

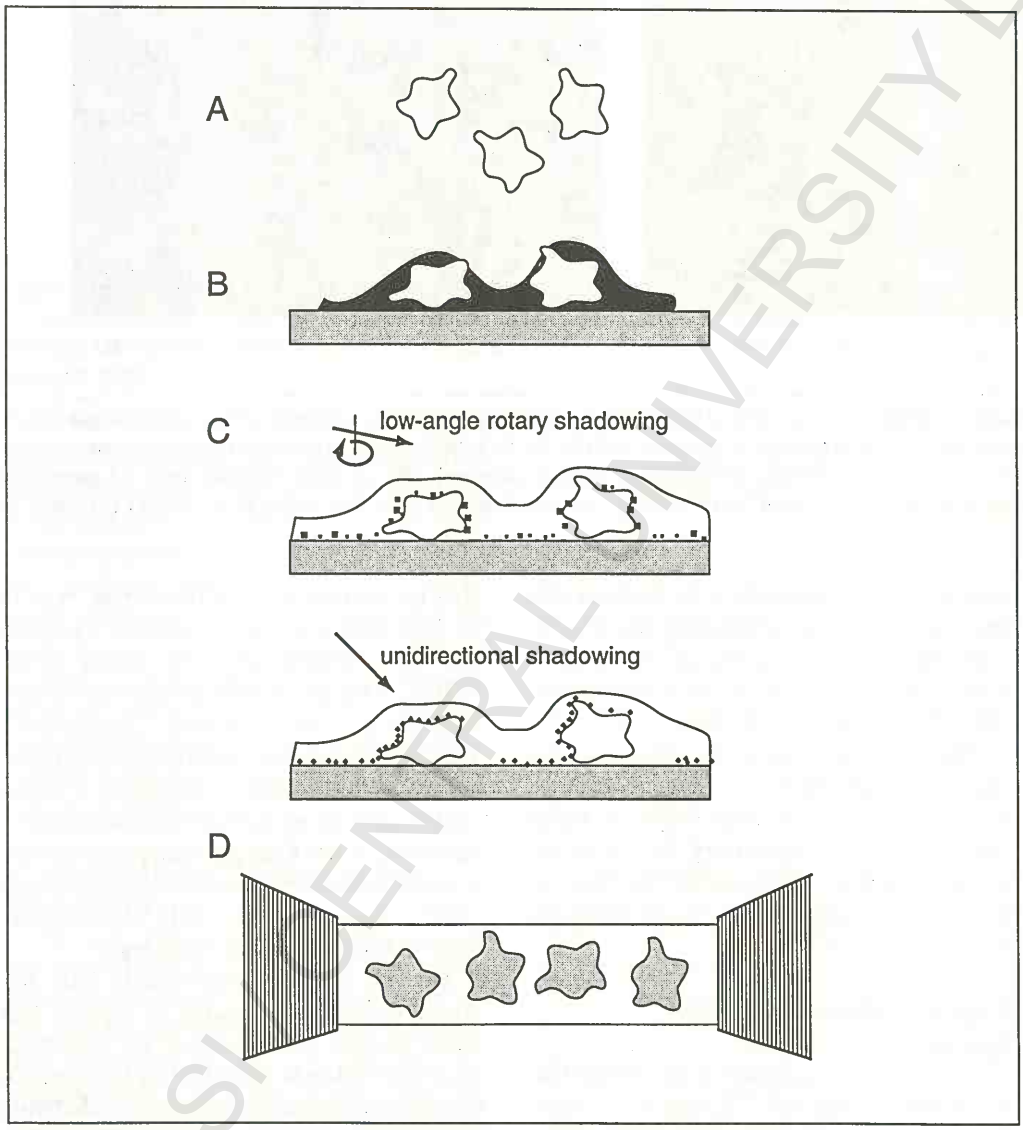

Figure 17.2.6 Preparation of protein molecules for EM. (**A**) Schematic drawing of protein molecules in solution. (**B**) Protein molecules prepared for EM by negative staining. The molecules are adsorbed to a thin carbon film substrate and embedded in negative stain. In drying, they have undergone variable amounts flattening. (**C**) Protein molecules prepared for EM by heavy-metal shadowing. The molecules are supported on a thin carbon film. The top diagram depicts rotary shadowing with platinum from a low angle; note the grain-free zone adjacent to the molecule. The bottom diagram depicts unidirectional shadowing from a 45° elevation angle. Note the smaller grain size and the grain-free shadow zone. (**D**) Protein molecules prepared for observation by cryo-EM. The molecules are embedded in a thin film of vitreous ice maintained at a temperature of approximately –180°C.

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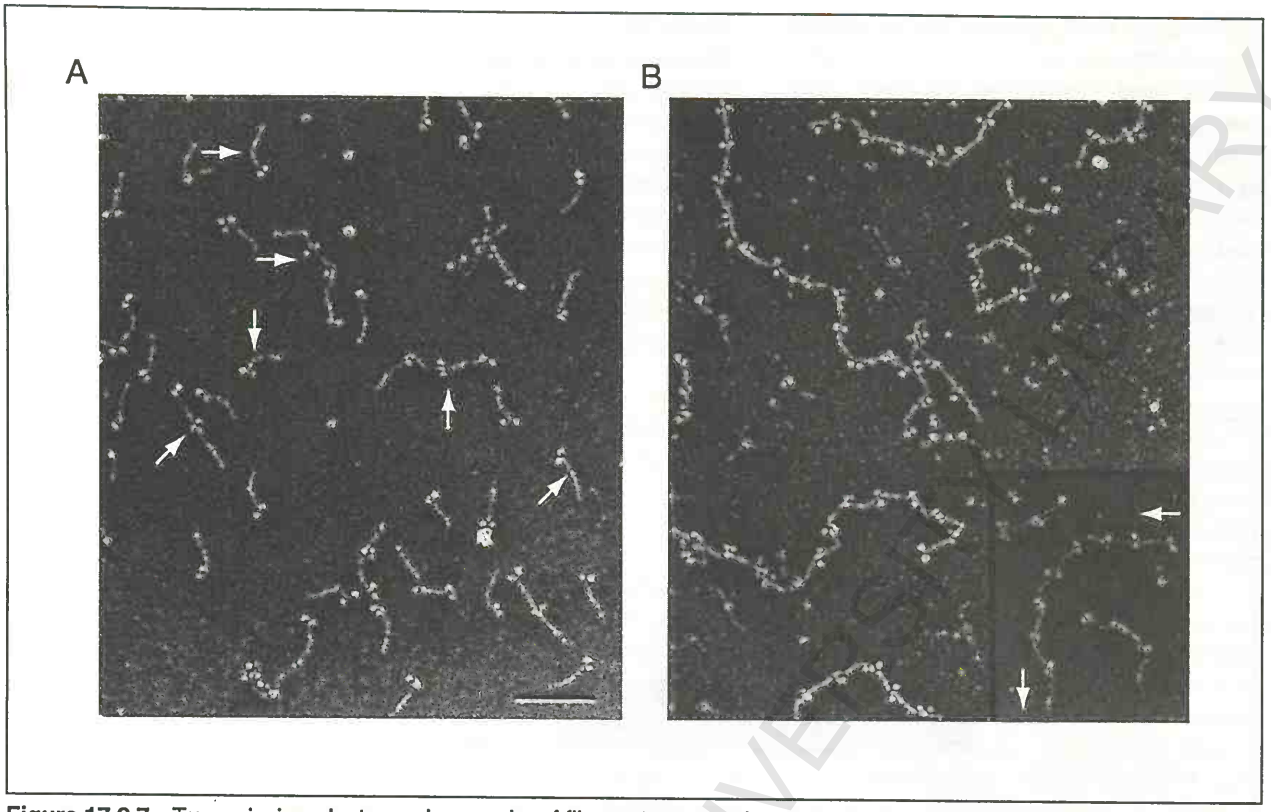

Figure 17.2.7 Transmission electron micrographs of filamentous proteins prepared by glycerol-spraying/low-angle rotary metal shadowing. The bar at the bottom of panel A is equal to 1000 Å. (**A**) At their first level of structural organization, nuclear lamin polypeptides form "myosin-like" parallel, unstaggered dimers (arrows). (**B**) At their second level of structural organization, lamin dimers assemble into linear head-to-tail polymers. Adapted from Heitlinger et al. (1991) courtesy of Dr. U. Aebi.

measured from the lengths of the shadows that they cast, and these micrographs may also be analyzed digitally to calculate topographic maps of molecular surfaces (Smith and Kistler, 1977; Thomas et al., 1991; Fuchs et al., 1995). On the other hand, images of rotary-shadowed specimens tend to be easier to interpret because they contain fewer lacunae where no metal grains reach. Low-angle rotary shadowing has proved particularly effective for the study of filamentous proteins (see discussion of Protein Filaments and Fig. 17.2.7).

Frozen-Hydrated (Vitrified) Specimens: Cryo-EM

In cryo-EM, the specimen is not dehydrated, but instead is preserved and observed in a thin film of ice (Fig. 17.2.6D). Provided that this film is thin enough and the freezing is fast enough, the water molecules are immobilized in an amorphous vitreous state, thus avoiding dehydration and/or physical disruption of the specimen by the formation of ice crystals. Once frozen, the specimen is inserted into a specially cooled specimen holder, or cryoholder, for transfer into the EM. Below about −110°C, ice does not sublime at a significant rate, even in the high vacuum of the EM column. Provided that the specimen remains below about −150°C, vitreous ice will not undergo a phase transition to a crystalline state. Commercially available cryoholders cooled by liquid nitrogen can maintain specimen temperatures of about −180°C, preventing both sublimation and crystallization. A few EMs are equipped to operate at liquid helium temperatures (approximately −269°C; Fujiyoshi et al., 1991), which confers some additional radiation-hardiness.

Because cryospecimens remain fully hydrated, there is good reason to suppose that native protein conformations are preserved. This expectation is supported by high-resolution electron-diffraction patterns recorded from ice-embedded catalase crystals (Taylor and Glaeser, 1974). A number of technical problems arise in connection with observing vitrified specimens. Heat transfer between the cryoholder and the microscope may cause the specimen to drift. Frozen specimens are liable to deposition of hoar-frost or other contaminants while in transit into the EM and even within the column, although additional cryoshielding

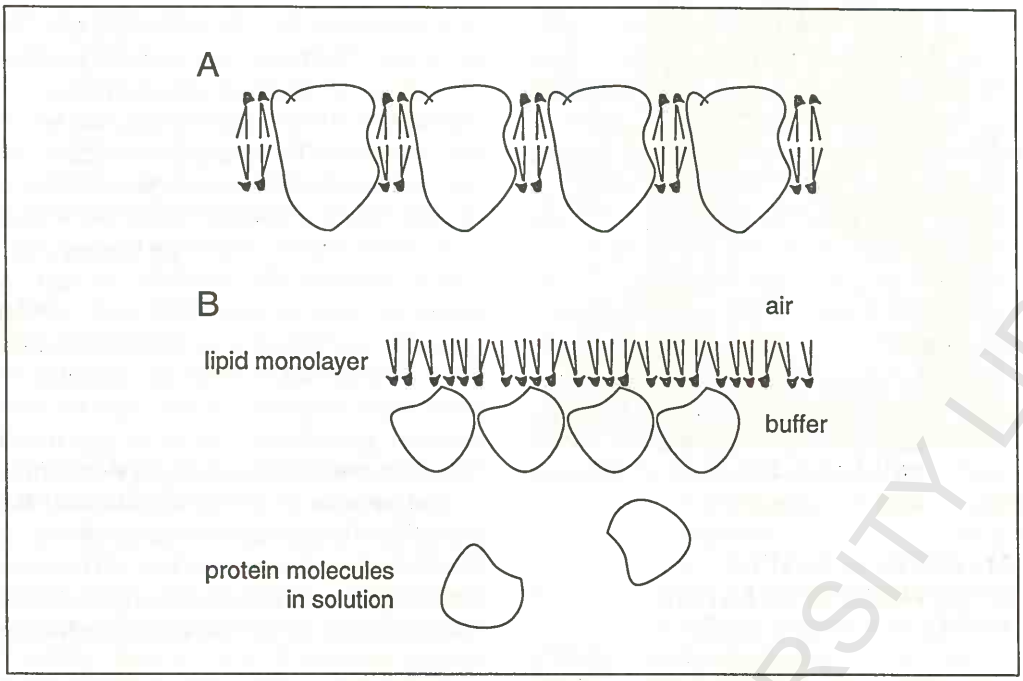

Figure 17.2.8 Formation of two-dimensional membrane crystals. (**A**) Intrinsic membrane proteins, initially solubilized in detergent, are reinserted into lipid bilayers as the detergent is removed by dialysis. (**B**) Extrinsic membrane protein with a specific affinity for a lipid monolayer (Langmuir-Blodgett film).

helps to minimize this effect. Because ice is not a good conductor, electric charge may build up on the specimen and distort the image. Also, vitrified specimens are acutely sensitive to electron irradiation (Conway et al., 1993) and, as noted previously, every precaution must be made to minimize the electron dose. These matters are still the focus of active developmental work; nevertheless, progress to date has made possible a wide range of experiments at resolutions that are already good and that continue to improve.

Crystallization of Proteins in Two Dimensions

Because monolayer crystals are so important for EM studies of proteins at high resolution (see discussion of High-Resolution Studies), a good deal of effort has been invested in their production. Occasionally, conditions that promote the formation of such arrays have been found serendipitously; e.g., Zn^{2+} ions promote monolayer crystal formation for soluble tubulin (Larsson et al., 1976). Two systematic approaches have been pursued, both based on the principle of confining the crystallands to a two-dimensional surface while maintaining their hydration (Fig. 17.2.8).

Intrinsic membrane proteins are an attractive class of molecules for study by EM (also see discussion of Membrane Proteins). The insolubility of these proteins adds to the list of parameters to be varied in a crystal screen. The sequence of steps that leads to crystallization—purification, concentration, and precipitation—must be expanded to include reinsertion into a lipid bilayer (Fig. 17.2.8A). Thus, the choice and concentration of detergent used to maintain solubility, as well as the choice and concentration of exogenous lipids for the reinsertion step, are also important. The temperature dependence of lipid mobility offers a possible mechanism for causing intramembranous proteins to undergo ordered aggregation. As the temperature falls below the threshold for a gel/liquid crystal phase transition, proteins may be "frozen out" of the ordered lipid phase. Based on this idea, a temperature-ramp method has been developed (Engel et al., 1992). A comprehensive overview of membrane-protein crystallization methods has been given by Kühlbrandt (1992).

An alternative crystallization strategy, applicable to extrinsic membrane proteins, involves the use of lipid monolayers formed at a (flat) air/water interface (Brisson et al., 1994; Fig. 17.2.8B). Such a monolayer presents a suitable surface for ordered aggregation of the proteins adhering from the underlying bulk phase. The protein of interest should have an

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affinity for the polar surface of such a monolayer. This affinity may either be natural or induced by appropriate chemical modification of the protein or the lipid head group (Kubalek et al., 1994; Celia et al., 1994). It should cause the protein to attach to the monolayer at a defined angle, leaving each molecule's azimuthal orientation about this axis as its the sole remaining degree of freedom, apart from the packing density. This crystallization technique has already been applied successfully in a number of systems, and excellent ordering and impressive resolutions have been obtained—e.g., annexins (Olofsson et al., 1994), α-actinin (Taylor and Taylor, 1994), and streptavidin (Avila-Sakar and Chiu, 1996).

MAPPING SPECIFIC COMPONENTS IN LARGE PROTEIN COMPLEXES

Once the three-dimensional structure of a large multiprotein complex has been determined to a resolution of 20 to 30 Å, it remains a major task to localize its various components within this frame of reference. A variety of approaches have been devised for different biological systems. They have in common the principle of seeking to modify the complex in some chemically defined way (e.g., the extraction of a loosely bound subunit or the binding of an antibody) and then visualizing the accompanying structural change. One serendipitous finding has been that when two well-defined—i.e., essentially noise-free—two-dimensional images or three-dimensional maps are compared, it is possible to detect structural differences that are smaller than the nominal resolution of the data. The explanation for this seeming paradox is that detectability is not the same as resolvability, but is a considerably more achievable goal. For instance, a point scatterer (infinitely thin) may be visualized by imaging techniques of modest resolution, although its representation is correspondingly blurred.

Difference Imaging: Depletion, Limited Proteolysis, and Complementation

One powerful approach to mapping subunits or domains is based on seeking to determine conditions under which one (or more) subunit or domain is selectively removed, while the remainder of the complex is left unchanged. Quantitative comparison of the modified complex with the original complex ("difference imaging") allows the vacated site to be localized. Two methods of accomplishing such removals are proteolytic dissection and selective extraction. Treatment with a specific protease may excise a domain or subunit (Carrascosa and Steven, 1978). Alternatively, extraction may be achieved by treating the specimen with a critical concentration of some denaturant such as urea or guanidine hydrochloride (Newcomb et al., 1993) or an extreme of pH (Yeager et al., 1994). Alternatively, alteration of surface charge by citraconylation (Aebi et al., 1977a) or similar reactions such as succinylation may prise particular subunits out of a complex by electrostatic repulsion. In any case, the biochemical groundwork has to be established before structural analysis becomes worthwhile. A third variation on this theme is to study the formation of complexes between the protein of interest and another protein that is known to bind to a particular subunit (Olson et al., 1993). This ligand then serves as a bulk marker for that subunit.

For any of the above treatments to achieve its intended purpose, it is important that the induced change should be strictly local. Thus, quantitative structural comparisons between images of treated and control (untreated) complexes are essential to guard against generalized structural changes, which potentially compromise the interpretability of such an experiment.

Immuno-EM: Labeling with Antibodies and Fab Fragments

Perhaps the most widely used variant of the marker-binding approach is visualization of complexes labeled with antibodies. It is essential that the specificity of the antibodies be clearly established by immunochemical methods. Immuno-EM labeling experiments may be performed either with intact antibodies or with monovalent Fab fragments (Aebi et al., 1977b; Wang et al., 1992; Porta et al., 1994). Both have their advantages. Whole antibodies may cause sizable immunoprecipitates to form if the protein happens to contain more than one copy of the epitope, and these precipitates may result in complicated images that are difficult to interpret. This problem does not arise with Fab fragments. On the other hand, precipitation may be advantageous in some situations. For instance, if the specimen is undesirably dilute and too fragile to survive centrifugation, it may be concentrated by immunoprecipitation (Newcomb et al., 1996).

With three-dimensional density maps calculated from cryomicrographs of antibody-labeled specimens, it turns out to be possible to localize the "footprint" of the antibody-binding

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site to within a few angstrom units, even if the map is limited to, say, 25-Å resolution (Steven et al., 1992; Smith et al., 1993). Similarly, the interaction between antibody and antigen can be modeled in detail by drawing on a priori information on the (conserved) structures of Fab moieties. The characteristic bilobed shape of an Fab may be readily detected in a cryo-EM density map at 25- to 30-Å resolution. Moreover, its orientation may be ascertained with high precision on the reasonable assumption that its point of contact with the protein marks the epitope.

Heavy-Metal Clusters: Small Site-Specific Labels

An important innovation capable of localizing sites on a protein complex has been the development of heavy-metal cluster compounds with organic moieties of defined functionality. Thiol-reactive clusters are particularly useful for labeling free cysteine residues, and site-specific mutagenesis techniques make it possible to insert cysteines into promising sites in a protein of interest. Single heavy atoms are hard to detect in the EM when attached to protein molecules, although they may be visualized when dispersed over a thin carbon film (Crewe et al., 1968). However, clusters of heavy atoms have enough scattering power to be visible, even when attached to proteins. The 11-atom gold cluster, undecagold, has been exploited successfully in several studies (Hainfeld, 1987; Milligan et al., 1990; Yang et al., 1994; Wagenknecht et al., 1995; Fig. 17.2.9), as well as a larger cluster of about 40 gold atoms called Nanogold (Gregori et al., 1997; Fig. 17.2.10). Nanogold may be visualized against a background of low-density negative stains (Wetzel and Baumeister, 1995), a property that expands its ease of use. Cluster compounds are now commercially available (see discussion of Resources and User Facilities), and as experience with them accumulates, this powerful labeling technique should become broadly applicable.

HIGH-RESOLUTION STUDIES

In the past few years, two EM-based approaches have progressed to the point of describing protein structures in near-atomic detail and revealing the detailed folds of polypeptide chains. The first is electron crystallography of highly ordered crystalline plates. These arrays are typically several microns or more in lateral extent, but very thin—ideally, only one molecule thick. To date, the two systems in which electron crystallography has advanced furthest—purple membrane (Grigorieff et al., 1996) and the light-harvesting complex (Kühlbrandt et al., 1994)—are both intrinsic membrane proteins, and encouraging progress has also been made in a number of other systems. This work is not confined to membrane proteins—a resolution of ~6 Å has been achieved with sheets of tubulin (Nogales et al., 1995), a cytoskeletal protein of great importance that has so far resisted attempts to produce three-dimensional crystals suitable for X-ray diffraction analysis.

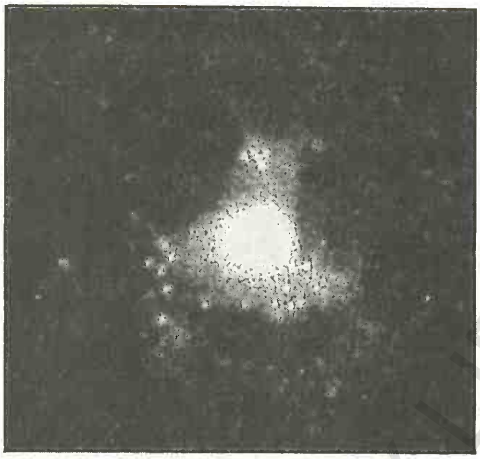

Figure 17.2.9 Dark-field STEM micrograph of a single ferritin molecule surrounded by gold-labeled Fab′ fragments of anti-ferritin antibodies. The specimen was unstained and supported on a thin carbon film. The small white dots are undecagold clusters and the large central white spot is the iron core of the ferritin molecule. Fab′ antibody fragments were covalently linked to undecagold at their hinge sulfhydryl, then incubated with ferritin and column purified. Courtesy of Dr. J. Hainfeld, Brookhaven National Laboratory.

In the second approach, three-dimensional density maps calculated by cryo-EM to resolutions of the order of 20 to 30 Å have been found to contain enough information to reveal the precise docking of subunits whose three-dimensional structures were separately solved to high resolution by other methods (Rayment et al., 1993; Stewart et al., 1993; Baker and Johnson, 1996). Thus, cryo-EM at moderate resolution serves as a bridge that allows the high-resolution information on individual subunits to be extended to the study of higher-order structures.

Electron Crystallography of Highly Ordered Two-Dimensional Arrays

Much of the electron crystallography methodology now available was pioneered in a series

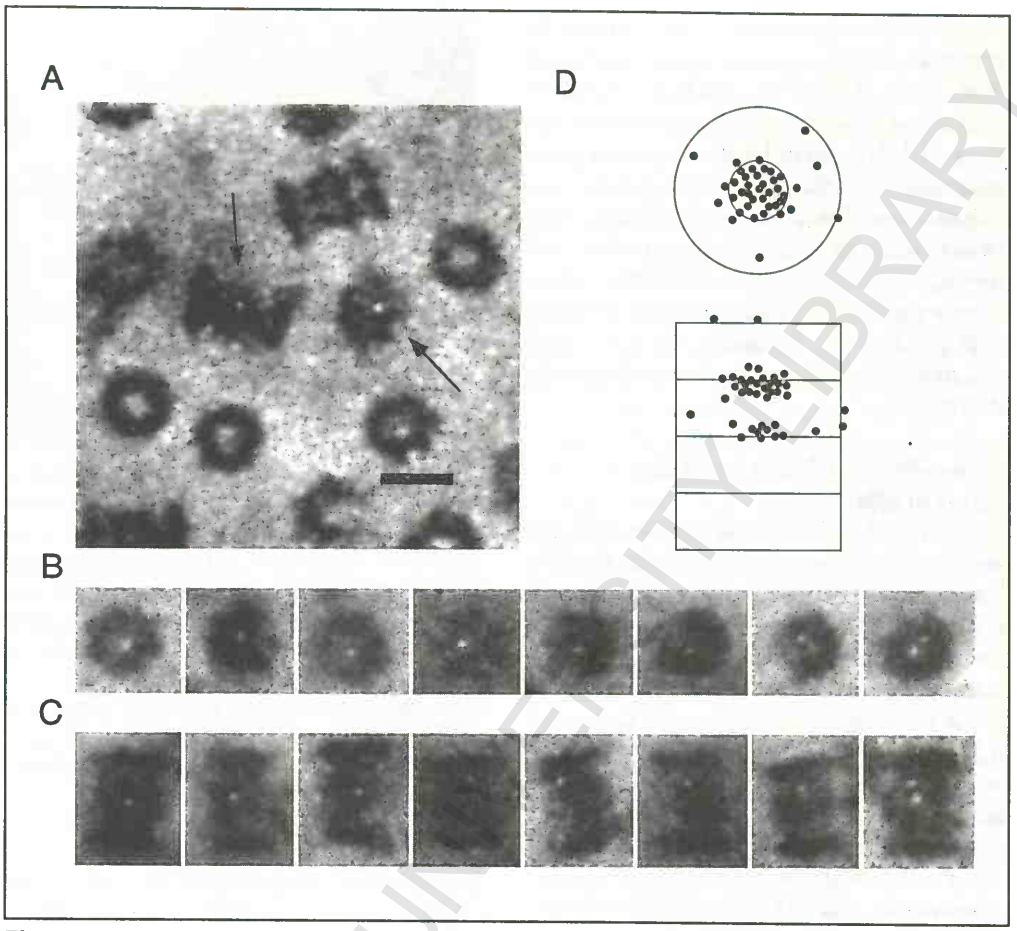

Figure 17.2.10 (**A**) STEM micrograph of proteasomes after binding Nanogold-labeled amyloid β protein, thus indicating its binding location. Labeling and binding were confirmed by electrophoresis, blots, and activity studies (Gregori et al., 1997). The 14-Å Nanogold clusters (arrows) are clearly visible with the low-density stain, methylamine vanadate (NanoVan; Nanoprobes), which enhances visualization of subunit detail. The bar is equal to 100 Å. (**B**) An array of top views of labeled proteasomes. (**C**) Side-views of corresponding labeled proteasomes. (**D**) Maps of the label sites recorded on many micrographs of the two views in panels B and C. Reprinted from Gregori et al. (1997), with permission, courtesy of Dr. J. Hainfeld, Brookhaven National Laboratory.

of studies of the purple membrane of *Halobacter halobium* (see Grigorieff et al., 1996, and the references in that paper). Under anerobic conditions, this bacterium expresses a pigmented protein called bacteriorhodopsin (bR), which functions as a light-driven proton pump to generate an alternative source of energy. In vivo, bR assembles into a crystalline monolayer that is exceptionally well ordered and thus ideally suited for study by crystallographic methods. The procedure relies on combining Fourier amplitudes (structure factors) collected from electron-diffraction patterns of single crystalline patches, with phases obtained from calculated Fourier transforms of low-dose electron micrographs (Unwin and Henderson, 1975). A single-projection view thus synthesized provides a central plane through the three-dimensional Fourier transform of the repeating unit (in this case, a trimer of bR). The remainder of the transform is filled in by tilting specimens to progressively higher angles and collecting diffraction patterns and images at each step. Each such view contributes another plane of the transform. Finally, the three-dimensional density map is calculated by inverse Fourier transformation. Because the accessible tilt range is limited and because data collection becomes progressively more difficult at higher tilt angles, the resolution of the density map is lower in the dimension perpendicular to the membrane. Nevertheless, electron-crystallographic analysis of bR has already been carried to a resolution of 3.5 Å in plane and ~5.5 Å in the third dimension (Grigorieff et al., 1996), which is sufficient to define the fold of the polypeptide chain and to locate the prosthetic group (retinal).

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The number of naturally crystalline protein monolayers that are as well ordered as bacteriorhodopsin is very small. To extend the range of specimens eligible for electron-crystallographic analysis, it is necessary to form artificial monolayer crystals (see discussion of Crystallization of Proteins in Two Dimensions). So far, the greatest success has been achieved with photosynthetic light-harvesting proteins (Kühlbrandt et al., 1994) and porins, a class of bacterial channels (Jap et al., 1991). However, promising results have also been obtained with several other proteins, including members of the aquaporin class of hydration channels (Walz et al., 1995) and gap junction connexins (Unger et al., 1997; see Fig. 17.2.11).

The latter study (of connexins) represents an important new departure in that the connexin arrays studied were not extracted from a source tissue but were expressed from a recombinant baculovirus in baby hamster kidney (BHK) cells, a cultured cell line that does not normally deploy gap junctions. Just as overexpression of soluble proteins has become a central element for structural studies, the expression and assembly of membrane proteins has become a vital objective for high-resolution studies of membrane proteins. As an added element of challenge, the requirement that expressed proteins should be inserted into membranes imposes a constraint on the amounts that can be expressed. For example, overloading the plasma membrane with foreign proteins may weaken the cell and impair its synthetic competence. If expressed membrane proteins precipitate into inclusion bodies, extraction and reconstitution may be difficult; perhaps incorporation into cytoplasmic storage membranes, if this can be contrived, may be a practically useful solution.

Hybrid Approaches: EM and X-Ray Crystallography

The complementarity between EM and X-ray crystallography (or any other technique capable of yielding subunit structures at high resolution) has been demonstrated in recent hybrid studies that combine the best of both approaches (Whittaker et al., 1995; Baker and Johnson, 1996). These studies serve as compelling role models for investigations of interactions that underlie protein-protein recognition phenomena and the assembly and functioning of protein complexes. In such studies, a high-resolution technique (to date, X-ray crystallography) is used to define the structures of individual molecules or subunits, and three-dimensional cryo-EM at 20- to 30-Å resolution is used to define the placement of these subunits in larger complexes. The pivotal point, which may not be evident, is that the cryo-EM density maps, despite their modest nominal resolution, contain sufficient information to allow the subunits to be docked to within a few angstrom units in their relative positions and orientations.

To come to terms with this seemingly fanciful proposition, it should be borne in mind that interpreting the cryo-EM map is not a structure determination per se, but rather is an exercise in pattern recognition for which much a priori information is available. In this context, it is worth bearing in mind that interpreting a crystallographic map is also in part an exercise in pattern recognition. The assumptions made include the following: the density distribution should represent a known number of polypeptide chains; these chains are continuous and unbranched; their amino acid sequences are (usually) known, which assists chain tracing; and familiar elements of secondary structure, such as α-helices, may be recognized at an early stage, thereby reducing the complexity of the problem. In this vein, interpreting a cryo-EM map of a complex of subunits of known structure hinges on whether there is enough information for the locations and orientations of these subunits to be identified correctly. It is sometimes argued that one only needs to determine six parameters per subunit—three Cartesian coordinates for its center of mass and three orientation angles. For this purpose, a limited amount of structural information should suffice. The only situation in which such correlation of crystallography with cryo-EM would be compromised would be if large-scale conformational changes were to accompany complex formation. The likelihood of such an eventuality has to be assessed on a case-by-case basis.

RESOURCES AND USER FACILITIES

The scanning transmission electron microscopy (STEM) facility at Brookhaven National Laboratory, Upton, Long Island, New York, is supported by the National Center for Research Resources (NCRR) of the National Institutes of Health (NIH). This facility operates two high-performance scanning transmission electron microscopes and specializes in STEM-based mass determinations and mass-mapping analyses. Its resources are available to appropriate user-initiated projects. The Brookhaven facility also has consi

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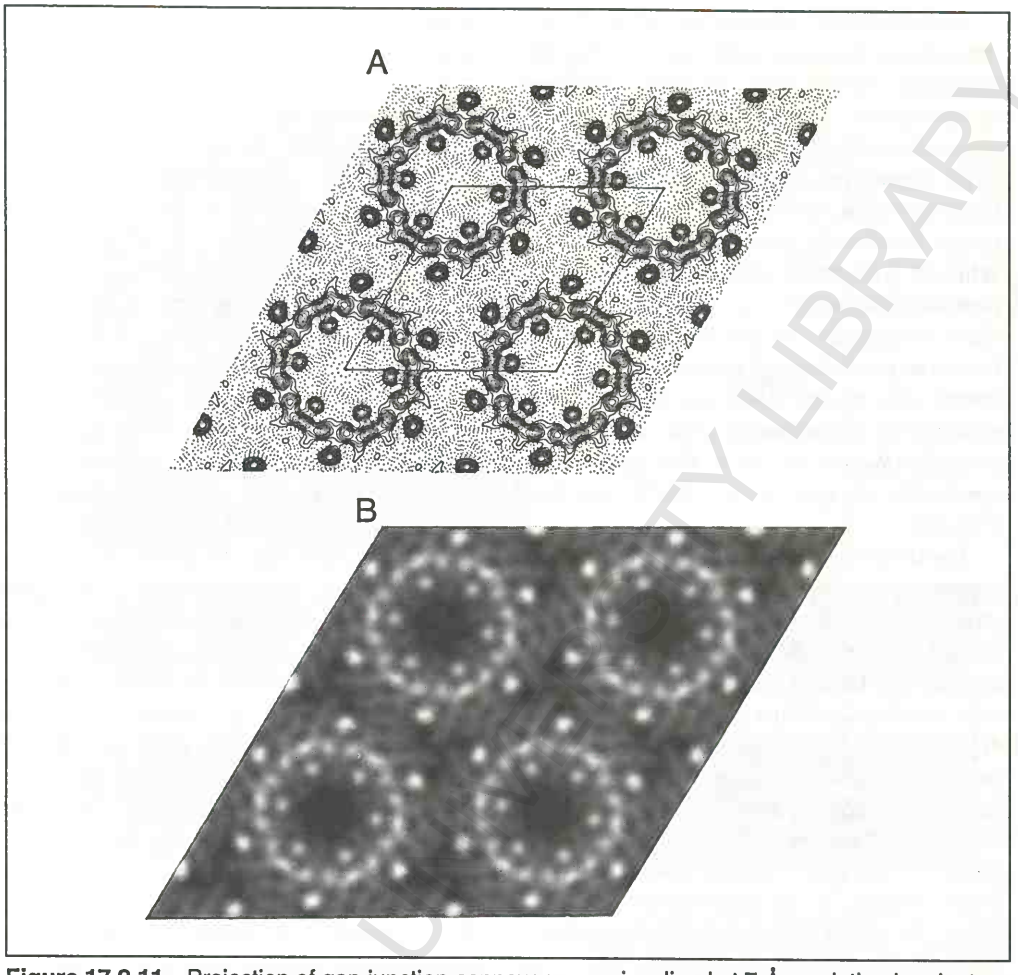

Figure 17.2.11 Projection of gap junction connexons, as visualized at 7-Å resolution by electron crystallography of ice-embedded specimens (Unger et al., 1997). The hexagonal unit cell contains two layers of hexameric rings of a rat connexin protein, truncated at its C terminus. This construct was expressed in BHK cells, where it spontaneously formed connexon plaques. There are four transmembrane α helices per subunit; two are oriented perpendicular to the membrane and are visible as the 10-Å disks of densities that lie, respectively, outside and inside the ring. The other two helices adopt a slanting orientation. As seen in projection, they overlap to contribute the features seen in the ring. (**A**) Contour plot. The side of the hexagonal unit cell is 95 Å. (**B**) Gray-scale representation. High densities are shown as white. Courtesy of Dr. M. Yeager.

beling molecules with heavy-metal clusters and in visualizing cluster-labeled molecules embedded in low-density stains. For more information see Internet Resources.

The National Center for Macromolecular Imaging at Baylor College of Medicine, also supported by the NCRR, has a 400-keV electron cryomicroscope and computer processing equipment to assist or collaborate with biological users in studying high-resolution three-dimensional structures of macromolecules. For more information see Internet Resources.

Heavy-metal cluster compounds for protein labeling experiments may be purchased from Nanoprobes (see *SUPPLIERS APPENDIX* for contact information; for technical questions contact Dr. Richard Powell, Research Director).

Image-processing software for the analysis of biological electron micrographs of proteins and complexes has been summarized comprehensively in a recent special issue of the *Journal of Structural Biology* (Carragher and Smith, 1996).

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Principles of Macromolecular X-Ray Crystallography

In the last 10 years there has been an astonishing increase in the number of protein structures determined by X-ray crystallography. As of September, 1996, there were some 4700 such structures in the Protein Data Bank, and the number is increasing exponentially. There is almost no limit to the complexity of the structures that can presently be determined by X-ray diffraction, provided that adequate ordered crystals are available. These advances have had a corresponding effect on concepts of modern biochemistry, where the profusion of three-dimensional structures has introduced a new degree of chemical sophistication into the discussion of the functions and interactions of biological macromolecules.

However, the technical nature of crystallography presents a formidable barrier to the biochemist who wishes to assess a crystallographic paper. How can the quality of the information coming from this technique involving crystals, computers, and X rays be critically evaluated? This unit does not attempt to explain all the details of crystal-structure determination, which are more adequately described in textbooks. It does, however, provide a brief overview of the general principles and methodology, as well as a more detailed discussion of the methods used to prepare a protein sample for crystallization trials and the various stratagems that are sometimes necessary to achieve this goal.

CRYSTAL STRUCTURE DETERMINATION BY X-RAY DIFFRACTION

There are a number of excellent reference works on macromolecular crystallography. These include early classic texts such as Blundell and Johnson (1976) and the more recent, comprehensive description of the principles of X-ray diffraction by Drenth (1994). There are several volumes of *Methods in Enzymology* (Wyckoff et al., 1985; Sweet, 1997) that provide more detail about advanced techniques. Consistent with the growing accessibility of protein crystallography to a broader community of scientists, there are also texts such as Jones et al. (1996) and Rhodes (1993) that are meant more for the biochemist or molecular biologist. Another recent text is by McRee (1993).

X-ray diffraction analysis of crystal structures is quite analogous to using a very high-power microscope that can see molecular shapes. The wavelength of the light used is usually in the vicinity of 1.5 Å, about the length of a carbon-carbon single bond, and the use of X rays with this wavelength permits, in theory, resolution of individual atoms.

In a microscope, a light beam strikes a specimen and the scattered light is brought to a focused image by a lens. In principle, X-ray diffraction functions in much the same way except that there is no X-ray lens to focus the X rays that are scattered by the electrons within the crystal. Therefore, it is not possible to directly obtain an image that can be interpreted. Instead the crystallographer measures the intensities of the scattered X rays from the crystal and uses these, together with some additional data described below, to compute an image.

Crystallographers use a crystal because the signal from a single molecule would be too weak to detect. The crystal acts as a three-dimensional diffraction grating and scatters the incident beam of X rays only in certain directions. But all the molecules in the crystal scatter together in these directions, and since there are $>10^{15}$ molecules, at least in most crystals, this enables the scattered signal to be recorded before the specimen is destroyed by radiation damage.

It can be demonstrated that this diffraction is the vector sum of the diffraction by the individual atoms of the unit cell, each having an amplitude that is related to the number of electrons and the size of the atom as well as a phase that is derived from the position of the atom within the unit cell. The sum of these individual contributions is a vector with an amplitude $|F(\underline{h})|$ and a phase $\alpha(\underline{h})$ where h is equal to (hkl) and h, k, and l are the Miller indices that define the diffraction maxima.

$F(\underline{h})$, called the structure factor (because it depends only on the structure of the scatterer), can be expressed in terms of the f_j, the individual scattering factors for the j atoms in the unit cell, and the atomic coordinates x_j.

$$F(\underline{h}) = \Sigma_j f_j \exp 2\pi i(\underline{h} \cdot \mathrm{x}_j) = |F(\underline{h})| \exp i\alpha(\underline{h})$$

By rotating the crystal relative to the X-ray beam, it is possible to record and measure the

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complete diffraction pattern of the crystal. The crystallographer can measure $|F(\underline{h})|$, which is directly related to the intensities of the diffraction maxima, but cannot experimentally measure the phase angle, $\alpha(\underline{h})$. These lost phases have to be recovered by some other method. When both the amplitudes and the phases of the scattered X rays are known, they can be combined to produce an electron-density map of the contents of the unit cell, the repeating unit of the crystal. The electron density (ρ) at any position *xyz* in the unit cell of the crystal is represented by the following equation (with *i* equal to the square root of −1).

$$\rho(x,y,z) = \Sigma_{hkl}|F(h,k,l)|\exp i\alpha(hkl)\exp -2\pi i(hx+ky+lz)$$

Data Collection

There are many methods available for measuring the intensities of the scattered X rays in X-ray crystallography. The two early methods involved film, which could measure an array of peaks simultaneously, and proportional or scintillation counters, which measured the data one spot at a time. Film has been replaced by imaging plates, which can be exposed to the scattered X rays, then rapidly read, erased, and reexposed. To reduce dead time, commercial imaging-plate detectors use two imaging plates so that one can be exposed while the other is being read. Multiwire proportional detectors have replaced single detectors, again permitting the measurement of many reflections simultaneously. A modern laboratory might employ either or both of these methods of data collection; for the future there is the emerging technology of charge-coupled devices (CCDs).

The laboratory X-ray source has traditionally been a rotating anode X-ray generator, usually with a copper target, which gives a peak of CuKα radiation of wavelength 1.54 Å. However, increasingly data are being collected using synchrotron X-ray sources at a few centers specifically designed for this purpose. Synchrotrons can provide X-ray intensities many hundreds or even thousands of times more intense than those attainable in the laboratory. They also have the advantage of providing a continuous band of radiation wavelengths from which a monochromatic beam of predefined wavelength can be selected for anomalous dispersion experiments (see discussion of Phase Determination).

Until recently, data for biological macromolecules were measured from crystals at ambient temperatures. However, lower temperatures reduce radiation damage to the crystal and much current data collection is carried out using so-called "cryo" techniques at temperatures close to that of liquid nitrogen. In this way, a single crystal will suffice to measure a complete data set.

Phase Determination

Having obtained crystals of a protein that diffract X rays to a reasonable resolution, the next step is to obtain an interpretable electron-density map, and for this one needs a reasonably accurate set of phase angles, $\alpha(hkl)$. Protein crystallographers generally use three methods to obtain phase information, two of which (multiple isomorphous replacement and anomalous scattering) involve heavy atoms, while the third uses a procedure called molecular replacement.

Multiple isomorphous replacement

The isomorphous heavy-atom replacement method, which was originally applied to the determination of phases for hemoglobin by Max Perutz and colleagues (Green et al., 1954), requires the attachment of a heavy atom to the protein in the crystal. There are enough sites of weak attachment on protein surfaces that it is usually sufficient simply to soak the crystals in a solution containing the heavy atom. An alternative procedure involves cocrystallizing the protein in the presence of the heavy-atom compound. The heavy atom will then also contribute to the X-ray scattering, and because it is electron-dense (with a large f_j), there will be a measurable difference in the intensities of the diffraction pattern. These differences can be used to locate the site of binding of the heavy atom. If several such crystals can be prepared with different heavy atoms binding to different sites on the protein, the results can be combined to calculate the phases of the protein structure factors. From these, an electron-density map can be computed. This map will contain errors resulting from the difficulties associated with the accurate measurement of small intensity differences. It will contain additional errors that are due to the imprecision of the phasing process, which in turn result from the lack of isomorphism between the native and heavy-atom crystals. Nevertheless, it is usually possible to identify regions that will fit the known sequence and to proceed to construct a model that will be consistent with the polypeptide backbone and side chains of the protein.

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Anomalous-scattering methods

A second method of phase determination also uses heavy atoms but takes advantage of the fact that atoms at wavelengths near their absorption edges will scatter X rays anomalously with a change in amplitude and phase. This anomalous scattering can be used for a single heavy-atom derivative, first to identify the binding sites for the heavy atom, then to compute the phases for the crystal as a whole. Although this procedure can be carried out at a single wavelength, the effects are much greater at wavelengths near or at the absorption edge of the anomalous scatterer. By measuring the diffraction intensities at wavelengths near and distant from the absorption edge, optimal intensity differences can be observed and used to solve the structure. The availability of tunable sources of X rays from synchrotron radiation has provided an effective means of doing this and has led to the development of the multiwavelength anomalous dispersion (MAD) procedures by Wayne Hendrickson and his colleagues, who used selenomethionine as a replacement for methionine to provide a suitable anomalous scatterer (Yang et al., 1990). The big advantage of the anomalous-scattering procedure is that the structure can be determined with data collected from a single crystal containing a suitable heavy atom.

Molecular replacement

When the structure of the protein is expected to be closely related to another known protein structure—e.g., in the case of a mutant protein that has crystallized in a different crystal form, or when the unknown protein has high sequence homology with another protein of known structure—this similarity may be used to obtain preliminary phase information about the unknown structure, which can then be refined. This method, known as the molecular-replacement method, is used in two stages—first to determine the rotation function and then to determine the translation function. The rotation function determines the rotation necessary to position the known molecule in the unit cell with an orientation corresponding to the unknown molecule. The translation function is then used to determine the position of the correctly oriented search molecule in the cell. In general this consists of stepping the search molecule through the unit cell, avoiding bad stereochemical contacts and looking for a location that will provide a reasonable agreement between the observed and calculated structure factors. A detailed description of the application of this method is beyond the scope of this chapter; interested readers may consult texts such as Drenth (1994) or the chapter by Tickle and Driessen (1996) for more information. Molecular replacement can be very powerful when the two molecules are not too dissimilar, and for molecules belonging to some superfamilies, such as antibody Fabs, it is now the method of choice. Several software packages exist for the molecular-replacement method. These include AMORE (Navaza, 1994), MERLOT (Fitzgerald, 1988), and CCP-4 (Collaborative Computer Project No. 4, 1994).

Resolution

Once the phases are known, an electron-density map can be calculated. The resolution of this map depends on the angular extent to which the intensity data have been measured. This is related to the spacing, d, of lattice planes within the unit cell according to Bragg's Law:

$$\lambda = 2d \sin\theta$$

where λ is the wavelength of the diffracted X rays and θ is the diffraction angle. Resolution in a protein structure is usually defined as the value of the d_{min} corresponding to the θ_{max} of the data set. For highly ordered small-molecule crystals that can diffract to the limit allowed by the wavelength of the X rays used—i.e., to $\theta_{max} = 90°$—the resolution would be $\lambda/2$ Å. Proteins have not yet been observed to attain this resolution, and crystallographers usually have to settle for something less.

Figure 17.3.1 shows the effect of resolution on an electron-density map for an α helix. It can be seen that at low resolution (e.g., 6 Å) there is no detail, but the helix takes the form of a rod of density. As the resolution increases (i.e., d decreases between 3.0 and 2.0 Å) the details of the main-chain and side-chain structures begin to appear. Individual atoms cannot be seen until the resolution is 1.0 Å or better, but based on the knowledge of peptide and amino acid shapes from small-molecule crystal structures it is possible to fit the map with a model based on the known sequence of the protein.

The ultimate resolution of protein data is determined by the degree of order in the crystal. Small-molecule crystals that do not contain large amounts of water usually can diffract to a resolution of better than 0.5 Å. However, protein crystals are inherently less ordered than this, partly because the protein structure itself has flexibility as a result of being a long polypeptide chain held together by weak forces—e.g., van der Waals' forces and hydrogen bonds.

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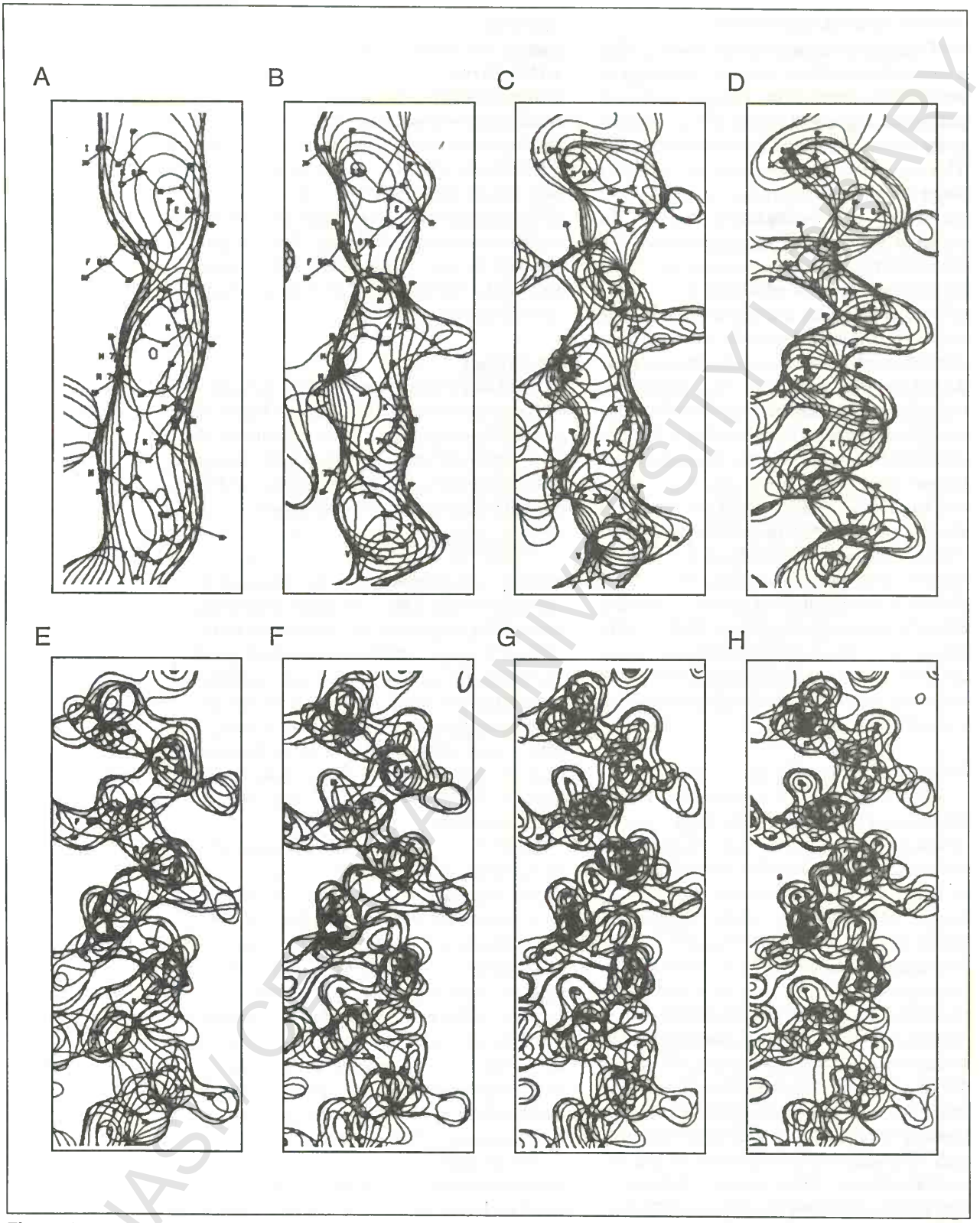

Figure 17.3.1 Calculated electron density for an α helix of myohemerythrin at resolutions of (**A**) 6.0 Å; (**B**) 5.0 Å; (**C**) 4.5 Å; (**D**) 4.0 Å; (**E**) 3.0 Å; (**F**) 2.5 Å; (**G**) 2.0 Å; and (**H**) 1.5 Å. Courtesy of Dr. Steven Sheriff, Bristol-Myers Squibb, Princeton, N.J.

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Also, the protein molecules are not packed identically in the crystal because there tend to be relatively few interactions between adjacent protein molecules; these are mostly weak and often they occur through intermediate water molecules. Finally, the spaces between the molecules are filled with large amounts of solvent, and these solvent channels tend to be quite disordered. Typically, a protein crystal will contain 50% solvent, the bulk of which is disordered. As a result, protein crystals do not often diffract beyond 1.8 Å, and the majority of protein crystals used for structural studies diffract in the range of 2.0 to 2.8 Å. It should be noted that since a protein crystal diffraction pattern does not just abruptly stop but gradually fades away with diffraction angle, the assignment of a resolution limit for a given crystal is to some extent subjective.

These limitations on resolution place limits on the accuracy of the structure that is being determined. For instance, water molecules are difficult to locate with precision in crystal structures where the resolution is worse than 2.5 Å. Also the lower resolution of the electron-density maps in protein crystals means that individual bonded atoms cannot be resolved. However, the knowledge from small-molecule crystallography about the dimensions of the amino acid components enables the crystallographer to fit the map and make reasonable assumptions about the positions of the atoms.

Let us assume that the heavy-atom phasing (see discussion of Phase Determination) has produced an electron-density map to a resolution of 2.5 Å. The next step is to fit a polypeptide chain with the correct sequence to this map. This can be done by first using a software package such as O (Jones et al., 1991) that will fit the polypeptide backbone automatically wherever the map quality is good enough to permit such interpretation. Once this is done, the next step is to identify sequence markers—i.e., those amino acids with large side chains such as Cys or Trp that can be recognized easily. The sequences between these markers can then be filled in. In this way a preliminary model for most of the structure can be assembled, although there are usually a few segments of polypeptide chain for which the map quality does not permit interpretation as a result of phasing errors or disorder in the protein structure. This preliminary model of the protein is now ready for the process of refinement.

Refinement

The initial model of the protein will contain significant errors resulting from the imperfect phasing of the experimental electron-density map. This model can be improved by a procedure that crystallographers call refinement. The aim of refinement is to minimize differences between the calculated and experimental intensities while at the same time optimizing the stereochemistry. The initial model can be used to compute the scattering amplitudes from the equation for structure factors (see discussion of Crystal Structure Determination by X-ray Diffraction). The calculated amplitudes $|F_c(\underline{h})|$ are compared with the observed $|F_o(\underline{h})|$, and the value of

$$\sum_{\underline{h}} \| F_o(\underline{h}) | - | F_c(\underline{h}) \|^2$$

is minimized, subject to some chemical constraints. There are a variety of software packages available for refinement, listed in Westhof and Dumas (1996). Since the number of diffraction data measured is usually less than the sum of the atomic occupancy and positional parameters to be determined, it is necessary to provide some additional information—e.g., about the allowed bond lengths, angles, and planarity of groups of atoms—in order to effect a meaningful refinement of the structure. This can be done either by placing constraints on the dimensions of groups of atoms or by restraining the dimensions to be within an ideal set of distances and angles. Reference dimensions are taken from the crystal structures of chemically similar groups determined by small-molecule crystallography where the resolution of the X-ray data is generally much higher than for proteins.

The residual ΔF will ultimately approach a limiting low value, which cannot be reduced by further refinement. This ultimate residual is due partly to errors in the experimental measurements of the values of F, partly to the failure to locate all of the ordered waters, and partly to the fact that the refined structure, with the constraints on stereochemistry, does not necessarily correspond exactly to the protein structure in the crystal. Also, it is difficult to model the dynamic nature of the protein molecule.

The progress of the refinement can be indicated partly by the value of the so-called R factor, given by:

$$R = \frac{\sum \| F_o(\underline{h}) | - | F_c(\underline{h}) \|}{\sum | F_o(\underline{h}) |}$$

and can vary from 0.4 to 0.5 (or from 40% to 50%, if expressed as a percentage) for an es-

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sentially correct but unrefined structure, to values as low as 0.12 (12%) for an exceptionally well-refined structure. Most well-refined structures produce R values <0.2 (20%). A low R value does not necessarily indicate that the structure is correct, and there have been several examples of partially incorrect structures that have yielded apparently low R values. Brunger (1992) has proposed a now widely used cross-validation procedure that involves omitting a certain percentage (10% is suggested) of the reflections in the refinement but using them to calculate an R^{free}, which will then provide an independent check of the validity of the structure. R^{free} is calculated in the same way as the standard R value, except that it is limited to these unrefined reflections.

The Ramachandran Plot

Another criterion used by crystallographers to assess their structures and to detect errors in the tracing of the polypeptide chain is known as the Ramachandran plot (Sasisekharan, 1962; Ramachandran et al., 1963). These authors demonstrated that the polypeptide backbone is not free to assume any conformation and proceeded to define systematically which regions of conformational space were allowed. The unallowed regions result from unacceptable contacts between the main-chain atoms, including the β carbon atom.

The Ramachandran plot provides a simple way of displaying the conformational data for a protein. The conformation at each α carbon atom can be defined by the dihedral angles ϕ and ψ, which correspond to rotations about the single bonds to the α carbon from the amide nitrogen and the carbonyl carbon, respectively. Varying ϕ and ψ will cause various atoms to make stereochemically unacceptable contacts. These combinations of ϕ and ψ will then represent high-energy conformations, and will for the most part be forbidden. This can be plotted on a planar diagram, the Ramachandran plot. This plot defines the parts of conformational space that are available to a polypeptide chain. The various secondary structures, of course, lie within the allowed regions—e.g., the α helix, the β sheet, and the collagen fold (see *UNIT 17.1*). The Ramachandran plot is used by crystallographers to demonstrate that the structure they have determined is stereochemically acceptable, and therefore appears in many structure papers. These days, most refinement programs have stereochemistry restraints built into them, so that it is rare to see a Ramachandran plot with a number of outliers.

PROCEDURES FOR CRYSTALLIZATION

Growing protein crystals suitable for structure determination has frequently been referred to as an art rather than a science. Although certain aspects of the method are empirical, there are many factors that can be controlled to maximize the probability of success. The overall approach is an iterative one. An initial set of crystallization conditions is tried, the outcome is observed, and if crystals do not immediately form, the conditions are then modified to take into account what was learned during the previous attempt. The following sections describe a general approach to obtaining protein crystals and include considerations to keep in mind throughout; examples from the recent literature and the authors' own experiences with HIV-1 integrase are included where appropriate.

Many excellent reference texts and reviews cover specific aspects of protein crystallization in more detail than will be presented here. These include McPherson (1982, 1990), Weber (1991), Carter (1992), Ducruix and Giegé (1992), and Gilliland and Ladner (1996). Valuable information can also be obtained from a number of Internet Web sites. The reader is referred to the Hampton Research Home Page (see Internet Resources) and the other Web sites described therein. Another useful reference is the Biological Macromolecule Crystallization Database (Gilliland et al., 1994; *http://ibm4.carb.nist.gov:4400/bmcd/bmcd.html*) where information is compiled on biological macromolecules that have been crystallized and the methods used.

Sample Preparation

The decision to attempt protein crystallization often begins with the observation that one has in hand a protein that is purifiable to relatively high homogeneity and shows promise in terms of solubility. Unfortunately, there are no absolute requirements for either homogeneity or solubility—what works for one protein may not be sufficient for another. Nevertheless, "the higher the better" is a good rule of thumb.

Assessing sample homogeneity

Protein homogeneity is usually assessed using denaturing gel electrophoresis—e.g., sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 10.1*). It is important to examine the protein over a range of concentrations—a concentrated sample (to the point of overloading the gel) will provide information on any contaminating proteins that

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are present in low amounts. A more dilute sample, on the other hand, may reveal that the protein is actually two or more proteins of very similar molecular weight arising, for example, from proteolysis. A good target range for purity is ~95%, although—as in organic chemistry—crystallization itself can be a method of protein purification. For example, the final step in the purification of cytochrome *c* peroxidase is repeated crystallization by dialysis against water (Fishel et al., 1987) and crystallization of the molybdenum-iron protein is part of the purification protocol for nitrogenase (Burgess et al., 1980). It is often useful to determine the nature of any contaminating proteins. If the contaminants are unrelated proteins, alternate or additional purification steps may be required. However, if the contaminating proteins are smaller fragments of the protein of interest (which may provide useful information regarding domain boundaries), the relative amounts of these can sometimes be manipulated. For example, in the case of recombinant proteins expressed in *Escherichia coli* that are susceptible to proteolysis during expression, the extent of protein degradation can sometimes be reduced by decreasing the cell density at which cells are induced, changing the concentration of the inducer, or dropping the temperature after induction (e.g., from 37°C to 20°C; also see *UNIT 6.1*).

Although SDS-PAGE is the method of choice to initially characterize protein homogeneity, a single band on a SDS-PAGE gel can be misleading. Heterogeneity can arise from a number of sources other than contaminating proteins, including post-translational modification, deamidation, amino acid side-chain oxidation, or multiple cross-linked species formed by intermolecular or intramolecular disulfide bonds. Advantage should be taken of the several analytical techniques that exist for evaluating protein heterogeneity. Attempts should be made, for example, to analyze the protein by nondenaturing (native) gel electrophoresis (*UNIT 10.3*) and by isoelectric focusing (IEF; *UNIT 10.2*). The critical role of information obtained by IEF was recently demonstrated for the complex between the human interferon γ receptor and interferon γ; only those protein fractions selected according to IEF gel criteria of homogeneity ultimately yielded crystals (Windsor et al., 1996). N-terminal sequencing (*UNIT 11.10*) and mass spectrometry analysis (Chapter 16) are also becoming routine as initial screens of homogeneity.

An important consideration to keep in mind throughout crystallization attempts is the redox state of any cysteine residues in the protein. If cysteine residues are present, it may be necessary to include dithiothreitol (DTT) or 2-mercaptoethanol in the protein buffer because the protein may become heterogeneous as a function of time as the protective reducing agents become oxidized. For example, analytical methods indicated that growth factor receptor–bound protein 2 (Grb2) formed covalent dimers as a result of cysteine oxidation, and it was necessary to include 5 mM DTT in the crystallization buffer to prevent their formation (Guilloteau et al., 1996). It is often useful to perform the initial protein characterization (e.g., SDS-PAGE and native gel electrophoresis) under both reducing and nonreducing conditions to determine if there is an effect on heterogeneity.

Another form of protein heterogeneity can arise from multiple conformations of the protein in solution. This may be a particular problem for flexible multidomain proteins where the domains may not be held in fixed positions relative to one another.

Amount of sample

Once it is clear that it is possible to obtain a protein of sufficient purity, the next factor to consider is quantity—i.e., how much protein is needed for initial crystallization trials? An initial screen of 50 different crystallization conditions is often the place to begin, and each condition requires ~3 µl of protein solution at a reasonable concentration (e.g., 10 mg/ml). This corresponds to 1.5 mg of purified protein and should be considered a bare minimum. A few milligrams of material allow a preliminary look at the protein's behavior in the presence of precipitating agents, but the first screening attempt is unlikely to produce large regular crystals. Enough material (10 to 20 mg) should be available for a few iteration cycles.

The second half of the "quantity" equation is that the 10 or 20 mg of protein must be in concentrated form. Most crystals are obtained using solutions with starting protein concentrations of 10 to 20 mg/ml, although crystals have been grown from solutions as low as 2 to 3 mg/ml (Kühlbrandt, 1988; Gilliland and Bickman, 1990). At the other end of the spectrum, crystals of the hemoprotein domain of $P450_{BM-3}$ were reportedly grown starting with a protein solution at 275 mg/ml (Boddupalli et al., 1992). It is difficult to know if 10 to 20 mg/ml reflects the optimal condition or simply the most commonly tried condition. Nevertheless, protein solutions in this concentration range appear likely to yield the best results.

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Sample concentration

Several methods exist for concentrating protein, most of which are familiar to the biochemist. Cases have even been reported where protein crystals have formed during concentration—for example, tRNA-guanine transglycosylase crystallized during concentration after the final purification step (Romier et al., 1996). A common technique is to remove excess protein buffer by filtration through a membrane (e.g., using ultraconcentrators under gas pressure or small concentration units that operate by centrifugal force; *UNIT 4.4* and *APPENDIX 3B*). However, not all proteins are amenable to this method, as some stick to or aggregate on the membrane surface. The authors have observed that this effect can sometimes be mitigated by changing the temperature at which the concentration is done; for example, certain deletion derivatives of HIV-1 integrase do not concentrate well at 4°C but behave nicely at room temperature. It should be noted that buffer components such as glycerol and detergents often also concentrate above these membranes. To ensure reproducibility, it is best to extensively dialyze a concentrated protein solution containing such additives against a standard buffer, remembering that it may take several days to reach equilibrium.

Another useful method of concentrating protein is ammonium sulfate precipitation (*UNIT 4.5*). A concentrated solution of ammonium sulfate or the salt in solid form is added to the protein solution and the resulting protein precipitate collected by centrifugation. The precipitated protein is then resuspended in a smaller volume of buffer, and it is a matter of taste whether the protein is used "as is" or after removal of the coprecipitating ammonium sulfate. One drawback of this method is that protein recovery may be low if the initial protein concentration is less than ~1 mg/ml (Scopes, 1982).

It may become apparent at this stage that it is not possible to concentrate the protein of interest beyond a certain threshold point or that when the protein is concentrated, it only transiently remains in solution before precipitating out. Protein engineering approaches may help circumvent these problems (see discussion of When All Else Has Failed).

Sample storage

The input of the biochemist is particularly important when the method of protein storage is selected because the decision involves not only knowledge of the protein's biophysical properties as a function of time but also its catalytic properties if it is an enzyme. Does the protein tolerate freezing at −80°C and subsequent thawing? Alternatively, is it stable for extended periods of time at 4°C? If the protein permits, it is convenient from the standpoint of reproducibility to freeze small aliquots of a concentrated sample that can be thawed as needed. This may require that glycerol or some other cryoprotectant be included in the protein buffer. Glycerol can subsequently be removed by dialysis prior to crystallization attempts, although this may not be necessary as glycerol is sometimes used as an additive in crystallization trials (Sousa, 1995). Storing the protein at 4°C avoids the perhaps traumatic step of freezing, but the possibility of sample variability as a function of time is then introduced.

Caution: Affinity-tagged proteins

It has become routine to engineer recombinant proteins to possess affinity tags attached to one of the termini; these tags permit rapid and selective purification on affinity columns. Such tags can take the form of another protein such as glutathione-*S*-transferase (GST; *UNIT 6.6*) or the maltose-binding protein (MBP), or can be as simple as a stretch of histidine residues that confers specific binding to metal affinity columns (*UNIT 9.4*). It cannot be assumed that these tags are innocuous. For example, the authors have observed that the presence of a 20–amino acid histidine-containing tag on the catalytic core domain of HIV-1 integrase negatively affects both its solubility and its ability to form crystals (A.B. Hickman and D.R. Davies, unpub. observ.). Similarly, Grishin et al. (1996) report that ornithine decarboxylase only forms crystals when the six histidine residues introduced at the N terminus are removed. Others (e.g., Zhang et al., 1994; Mol et al., 1996; Prodromou et al., 1996) have successfully crystallized proteins that retain tags, but in general these tags are short peptides of six to nine amino acids. As the inclusion of a protease cleavage site between the tag and the protein allows for tag removal during or after purification (Craigie et al., 1995; Grishin et al., 1996; Guilloteau et al., 1996), this precaution should be seriously considered. Exposure of the protein of interest to a protease may bring with it new problems such as proteolysis at internal sites (Guilloteau et al., 1996) or heterogeneity as a result of incomplete removal of the tag. Nevertheless, it may be worth the time invested to surmount these hurdles prior to undertaking crystallization attempts.

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Physical Characterization of Protein Sample

If it has been possible to concentrate the protein to crystallographically useful levels, the next points to address are the multimeric or aggregation state of the protein and the minimal buffer components required to keep the protein soluble. Although these questions will be considered separately, it should be apparent that the results are intertwined; when the protein buffer is modified, the biophysical properties of the protein may also change. Information on the relationship between these two considerations is often invaluable in understanding and interpreting the results of initial crystallization trials.

A protein's multimeric or aggregation state in solution is an important predictor of success in forming crystals. The tendency of a protein to form aggregates or high-molecular-weight clumps is easy to evaluate when these aggregates are insoluble and precipitate out of solution. However, aggregated protein can also remain in solution as soluble high-molecular-weight material. If this material is heterogeneous—i.e., the aggregates are irregular in shape and size—it is unlikely that a regular crystal lattice containing reproducible individual units will form. It is crucial to determine if such aggregates are present and, if so, to devise methods to avoid their formation or to remove them prior to crystallization. Useful methods for determining the multimeric state of a protein in solution include gel-filtration chromatography (*UNIT 8.3*), dynamic light scattering, and analytical ultracentrifugation (*UNIT 7.5*).

Gel filtration chromatography

Gel filtration is a commonly used chromatographic step during protein purification; it separates biomolecules according to molecular size (for review see Stellwagen, 1990, and *UNIT 8.3*). The chromatographic support is comprised of porous beads and the diameter of these pores dictates the range of molecular weights over which proteins of different sizes are resolved. Information can be obtained about the protein multimeric state(s) if the column is calibrated using a set of appropriate molecular weight standards, although nonspecific adsorption and protein shape can have unpredictable effects on these results. The extent of aggregation in a sample intended for crystallography experiments can also be readily determined by measuring the proportion of material eluting in the column void volume. Companies that market fast protein liquid chromatography (FPLC) instruments often sell prepacked columns capable of high-resolution separation. Small-bore columns are also available to allow the analysis of very small samples—e.g., 10 to 50 µl. If a sample does contain aggregated material, gel filtration on a preparative scale can be a valuable technique for removing the high-molecular-weight material prior to crystallization trials.

Dynamic light scattering

Dynamic light scattering is another method that yields information about protein multimeric state. This technique affords the advantage of doing the analysis at the protein concentration of interest—unlike gel filtration, in which sample dilution is de rigeur (for review see Ferré-d'Anaré and Burley, 1994). In this method, a laser beam scattered by the sample is observed at a 90° scattering angle for a period of several seconds. The effective hydrodynamic radius of the scattering protein molecules in solution can be determined via the autocorrelation function of the scattered light intensity variation during the observation period. If the sample is monodisperse, the observed data can be interpreted in terms of a single hydrodynamic radius and the molecular mass of the scattering species thereby calculated. As an example of the usefulness of light-scattering data, Bentsen et al. (1996) reported that light-scattering measurements on phosphoribosylpyrophosphate synthetase and its complexes showed a direct correlation between polydispersity, the time required for crystals to appear, and ultimate crystal quality.

Analytical ultracentrifugation

Analytical ultracentrifugation can be used to derive information about the shape of molecules, molecular masses of species in solution, and thermodynamic parameters relating to their association properties (for reviews see Hansen et al., 1994, and Hensley, 1996). In sedimentation-equilibrium experiments, a protein sample is centrifuged at low speeds such that the rate of transport of molecules by sedimentation is balanced by the rate resulting from diffusion; under these conditions, the distribution of molecules at equilibrium can be measured and molecular masses calculated. Thus, for a well-behaved protein, it is possible to determine if the protein is present in solution as a monomer or a higher-order multimer and the appropriate association constants can be derived. For example, in the case of the catalytic core domain of HIV-1 integrase with an introduced point

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mutation (F185K), sedimentation-equilibrium studies demonstrated that the protein was a monodisperse dimer over a wide concentration range and therefore a suitable crystallization target (Jenkins et al., 1995). Since the method involves fitting experimental data to an assumed mathematical model, meaningful results can only be obtained when the analysis is performed over a range of conditions to obtain a unique fit to the possible variables. Unfortunately, this has the drawback of being both sample- and time-consuming.

Choice of buffer components

As indicated earlier, the biophysical state of a protein in solution may be dependent on the properties of the buffer used. As crystallization attempts involve changing the protein environment in a controlled manner, often by the addition of precipitating agents, keeping the concentrations and number of buffer components to a minimum allows the precipitant solution to dominate the properties of the resulting mixture. As most proteins are not soluble or stable in water alone, the usual buffer components that should be evaluated include: the buffering agent itself and the pH to which it is adjusted; protective compounds such as dithiothreitol, 2-mercaptoethanol, or EDTA; salts, if these are needed to keep the protein in solution; and any other agents required to stabilize the protein. A series of experiments to evaluate the effect of removing these components or decreasing their concentration can be performed using a concentrated protein solution with a full complement of buffer components (usually remnants of the last protein purification step), and then removing each by slow dialysis. It should be determined if changes in the buffer cause corresponding changes in the biophysical state of the protein or its homogeneity as defined by the previously described battery of techniques. In such a way, it is possible to define the minimal set of buffer components necessary to keep the protein in a homogeneous state for the expected duration of a crystallographic study.

First Crystallization Attempts

In essence, methods commonly used to coax protein crystals to form involve the very gentle approach to conditions of supersaturation (McPherson, 1985, 1990). In theory, once supersaturation is reached, if conditions are just right, the protein will gradually crystallize out of solution rather than fall out in the undesired form of an amorphous protein precipitate. Slow physical processes that can be exploited to drive a protein solution to supersaturation include evaporation, vapor diffusion, and dialysis.

The vapor-diffusion technique is probably the most widely used method for growing protein crystals. It involves the suspension of a drop of protein solution above a larger volume of a solution of the precipitant in a sealed chamber. Customarily, the droplet, ~3 µl in volume, is pipetted onto a siliconized coverslip, an equal volume of the well solution added and mixed, and the coverslip inverted and sealed with vacuum grease or oil over the well solution. Equilibration by vapor diffusion of the volatile species results in equalizing the vapor pressure over the well and the drop. This can produce conditions of protein supersaturation in the drop, which sometimes result in crystallization. Typically, the experiment is performed in 24-well tissue culture plates ("crystal trays"); the clear coverslips permit the visualization of the drop contents at regular intervals using a microscope. More detailed descriptions of this method can be found in Ducruix and Giegé (1992) and McPherson (1990).

A complete investigation of all the possible variables—e.g., different precipitants, temperatures, pHs, and concentrations—would involve unachievable numbers of experiments. Attempts to reduce this number by factorial procedures have been made (Carter and Carter, 1979), but they have been only partly successful. However, the empirical data obtained from thousands of proteins and protein-nucleic acid complexes have indicated the most popular and successful methods (Gilliland et al., 1994). The commercial availability of screening kits containing likely precipitants for protein crystallization (e.g., from Hampton Research; see *SUPPLIERS APPENDIX*) significantly reduces the number of solutions to be prepared. Thus, a reasonable place to begin is with a concentrated protein sample and a screening kit containing ~50 precipitant solutions.

Other methods that can be used to screen crystallization conditions (e.g., sitting drops, dialysis, and batch methods) are reviewed in McPherson (1982); gel methods are described in Robert et al. (1992).

Modifying Screening Conditions

As it is impossible to predict the rate at which crystals might form, it is important to monitor the processes occurring in the protein drops as equilibrium conditions are approached. The first set of observations should be obtained as soon as the crystal trays are set up. The drops should be carefully examined

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under the microscope. Are the drops clear or have precipitates formed? Is there phase separation of the protein in the form of oil-like droplets? Is there "extraneous material" in the drops that should not be mistaken for crystals at a later date?

The crystal trays should be reexamined within the next 24 hr and periodically thereafter. Each drop should be thoroughly examined at the bottom where heavy precipitate (or crystals) may settle, around the edges where equilibration may be the fastest, and against the surface of the coverslip, which can provide nucleation sites for crystals. If crystals do not form under the initial conditions, then careful observations can guide the rational modification of subsequent conditions. What is the relative proportion of drops remaining clear to those with precipitates? A large number of clear drops may suggest that the protein was not concentrated enough or that the amounts of precipitants should be increased. Alternatively, if most conditions give rise to heavy precipitates, the protein concentration should probably be decreased or the well solutions diluted. Extensive protein precipitation can also be caused by the initial two-fold dilution of buffer components upon mixing the protein with the precipitant solutions, if protein solubility is particularly sensitive to these. It should also be considered whether there are general trends across the range of precipitating agents that suggest certain compounds lead to protein precipitation while others tend to keep the protein soluble. Similarly, one should ask whether there is an apparent effect caused by pH or a particular salt. Remembering that the goal is to slowly approach supersaturation and induce the formation of crystals rather than precipitate, it is often clear which precipiting agents will be useful and how their concentration should be adjusted in subsequent crystallization trials. It should be noted, however, that crystals sometimes grow in a drop in which an amorphous protein precipitate initially formed. Furthermore, initial precipitates may disappear over time as equilibrium conditions are reached in the drop. Thus, precipitates are not necessarily a cause for undue concern.

Perhaps one of the most difficult assessments in the initial stages of crystallization trials is to judge the nature or promise inherent in a particular precipitate—there are "good" precipitates and then there are "bad" precipitates. Particular attention should be paid to how heavy the precipitate is and its color. It should also be determined if a precipitate is shiny or appears to have distinct edges, which may be indicative of tiny crystals rather than amorphous precipitate. Unfortunately, not much replaces experience—the investigator's own, and if available, that of a seasoned crystallographer.

In subsequent crystallization experiments in which the amounts or the nature of the components of the precipitant solutions are changed, another possibility is to supplement the well solution with various additives. The composition of the well solution, by virtue of its larger volume, will control the approach to vapor-pressure equilibrium. If salts are present in the protein buffer, they can be added to the well solution to control the ultimate volume of the drop. Under certain conditions, salts may also affect the rate of approach to equilibrium (Luft and DeTitta, 1995). It may be desirable to supplement the well with a reducing agent such as dithiothreitol (DTT) or 2-mercaptoethanol if previous experiments have demonstrated that a reducing environment is required to keep the protein in a homogeneous state. Inclusion of such reducing agents can have marked effects on crystallization. For example, the authors have observed an absolute requirement for 5 mM DTT in the crystallization of the core domain of HIV-1 integrase (Dyda et al., 1994); others have reported similar results for other proteins (Guilloteau et al., 1996).

Once microcrystals or small crystals are observed, it is usually necessary to optimize conditions to obtain crystals of sufficient size and quality for data collection. This is done by varying such parameters as the concentrations of the precipitant components, the types of components (e.g., the nature of the salts or the molecular weights of polyethylene glycols), the protein concentration, and the pH. Obtaining suitable crystals may also require resorting to other methods such as sitting drops (McPherson, 1990), microseeding, or macroseeding (Stura and Wilson, 1992).

When All Else Has Failed: The Use of Protein Engineering

At some point during the isolation or characterization of a particular protein, it may become clear that the protein persists in being insoluble and polydisperse. On the other hand, a protein that appears to fulfill all the criteria for being well-behaved in solution may adamantly refuse to form crystals. One method that has enjoyed some success in circumventing these problems is the use of antibody complexes (for reviews see Laver, 1990, and Kovari et al., 1995). Alternatively, instead of modify-

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ing the protein's environment, it is possible to modify the protein itself. The authors' experience with HIV-1 integrase, an essential enzyme in the retrovirus infection cycle, illustrates a number of protein-engineering approaches to modifying a protein in order to obtain a version amenable to concentration and crystallization.

Crystallizing protein domains

Wild-type HIV-1 integrase as recombinantly expressed in *E. coli* is relatively insoluble (~1 mg/ml in buffers containing 1 M NaCl) and tends to aggregate, forming high-molecular-weight material that either elutes in the void volume of a gel-filtration column or precipitates out of solution. A series of deletion mutants, or smaller versions of the protein, was examined to determine if any of these was more soluble that the full-length protein. Limited proteolysis experiments demonstrated that when integrase was exposed to trypsin, chymotrypsin, or V8 protease, it was converted to a series of smaller products as visualized by SDS-PAGE (Engelman and Craigie, 1992). Notably, a prominent protein of about half the molecular weight of the full-length protein was formed, which was resistant to further degradation. When the N-terminal sequence of this smaller protein was determined, it was clear that it represented the middle portion, or core, of the protein from which the two termini had been removed. Such protease-resistant protein fragments may be useful crystallographic targets as they are likely to represent distinct folded domains of the protein that are relatively compact (and hence relatively inaccessible to proteases). The approach of using limited proteolysis and then defining distinct domains by N-terminal sequencing or, more recently, mass spectrometry (reviewed in Cohen, 1996; also see Chapter 16) has been successful in many cases.

In the case of HIV-1 integrase, limited protease digestion experiments defined three protein domains: N-terminal, C-terminal, and central domains. All three domains were then separately expressed in *E. coli* and their solubility examined. While the central domain was no more soluble than the full-length protein, the C-terminal domain by itself was extremely soluble (Engelman et al., 1994) and could be readily concentrated to 20 mg/ml. Thus, the solubility of a full-length protein is not a good predictor of the solubility of its distinct domains. The structure of the C-terminal domain of integrase, which possesses the nonspecific DNA-binding properties of the intact enzyme, was recently determined by NMR spectroscopy (Eijkelenboom et al., 1995; Lodi et al., 1995).

Although probably not generalizable, an interesting example of the proteolysis approach to crystallizable domains was recently reported (Prodromou et al., 1996) in which hanging drops containing yeast Hsp82 chaperone protein were observed to be contaminated with fungus. Crystals of full-length Hsp82 that normally would have been obtained under these conditions did not form. However, a few days later, a new crystal form was observed. The crystals were dissolved, and N-terminal sequencing indicated that proteolysis, presumably by fungal enzymes, had generated a truncated form of the protein consisting of the N-terminal domain, which readily crystallized.

The importance of being compact

Many proteins that are of interest from a structural point of view are composed of multiple domains. It is becoming clear that when attempting crystallization of an isolated protein domain, ultimate success can be crucially dependent on the residues chosen to define the domain boundaries. Thus, it is fairly common to generate a series of protein constructs of slightly different lengths and to investigate the crystallizability of each protein. The need for such an approach was demonstrated in the cases of the N-terminal region of cartilage oligomeric matrix protein (Efimov et al., 1996), and the transcriptional coactivators dTAF42 and dTAF62 (Xie et al., 1996). Truncation may also be necessary for other reasons. For example, the N terminus of the extracellular domain of the human interferon γ receptor is particularly susceptible to proteolysis (and hence heterogeneity); crystals were only obtained when eight amino acids were removed from the N terminus (Windsor et al., 1996).

Mutation of nonessential cysteine residues

During isolation of the separately expressed C-terminal domain of HIV-1 integrase, it was observed that concentrated protein precipitated out of solution, an effect readily reversible by the addition of dithiothreitol (DTT). Examination of the amino acid sequence revealed a single cysteine residue, suggesting that intermolecular cross-linking might be responsible for aggregation and subsequent precipitation. Accordingly, the cysteine residue was mutated to serine; the mutated protein remained soluble and no longer aggregated in solution. It was verified that the Cys-to-Ser mutation did not affect the DNA-binding properties of integrase.

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This approach of mutating nonessential cysteine residues to prevent aggregation is generally applicable. For example, the N-terminal domain of the Mu phage transposase contains a cysteine residue that was mutated to leucine prior to structure determination by NMR spectroscopy (Clubb et al., 1996).

Site-specific mutagenesis

Integrase activity assays demonstrated that the region of structural interest (the enzyme active site) was likely to reside in its central core domain (Bushman et al., 1993). Unfortunately, this domain proved to be as insoluble and as susceptible to aggregation as the full-length protein. Thus, a novel approach to improving solubility was undertaken (Jenkins et al., 1995). The hydrophobic residues in the central core domain were selected for site-specific mutation according to two criteria—(1) where an isolated hydrophobic residue occurred by itself within the amino acid sequence, it was mutated to a charged lysine residue, and (2) where two or more hydrophobic residues clustered in the primary sequence, they were collectively mutated to alanine residues. Altogether, 29 sets of mutations were introduced by PCR mutagenesis (see Cormack, 1997), the proteins expressed in *E. coli*, and cell-lysis experiments devised to rapidly screen for improved solubility. Three mutated proteins were identified that were more soluble than the unmutated core domain as assessed by initial solubility after cell lysis, and these were studied further. One of these proteins, in which a single phenylalanine residue was mutated to lysine (F185K), was subsequently demonstrated to be soluble to at least 20 mg/ml, dimeric and monodisperse under all conditions examined, and fully active (Jenkins et al., 1995). The introduction of this mutation was directly responsible for the subsequent crystallization and structure determination of the catalytic core domain of integrase (Dyda et al., 1994). Thus, surprising as it may seem, a single amino acid change can be sufficient to convert a protein that is completely unsuitable for crystallographic attempts to one that readily crystallizes. The problem, of course, is identifying such a mutation. In other successful structure-determination efforts, "magic" mutations have been introduced by accident, as in the case of polymerase chain reaction (PCR) artifacts during cloning of the bacterial chaperonin GroEL (Braig et al., 1994). In other cases, sets of site-directed mutants have been generated for different reasons than those used for HIV-1 integrase. For example, to increase protein yield during purification, *Staphylococcus aureus* dihydrofolate reductase was deliberately engineered to be more soluble; the protein containing two introduced point mutations readily crystallized (Dale et al., 1994).

To summarize, protein engineering can be applied to problematic crystallographic targets in a number of ways. Compact domains can be defined by proteolysis and subsequent identification by N-terminal sequencing (*UNIT 11.10*) or mass spectrometry (Chapter 16). Further trimming of the domain boundaries may be required to yield a tractable protein. Cysteine residues that may cause intermolecular cross-links can be removed. Furthermore, site-specific mutations can be introduced that may dramatically affect a protein's solubility, multimeric properties, and crystallizability.

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INTERNET RESOURCES

www.hamptonresearch.com

Hampton Research Home Page; discusses aspects of protein crystallography; contains links to other Web sites.

Contributed by Alison B. Hickman and David R. Davies
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Crystallization of Soluble Macromolecules

X-ray crystallography has evolved into a very powerful tool to determine the three-dimensional structure of macromolecules and macromolecular complexes. Developments in computational methodologies, crystal data collection procedures, and recombinant DNA technology have resulted in a near exponential pace for structure determinations over the last century. The major bottleneck in structure determination by X-ray crystallography is the preparation of suitable crystalline samples. Advances in procedures for macromolecular crystallization, together with the availability of commercial screening kits, have made it relatively straightforward to set up screens for crystallization in a conventional molecular biology or biochemistry laboratory.

The following unit outlines the steps required for the crystallization of a macromolecule starting with a purified sample. The starting macromolecule must be purified and homogeneous (≥95% pure by SDS-PAGE; *UNIT 10.1*), and can be at a fairly dilute concentration (≤1 mg/ml). The macromolecule should be in a standard nonphosphate buffer between pH 5.5 and 8.0, containing no detergent and a fairly low salt concentration (preferably <100 mM NaCl, and no higher than 250 mM). A reducing agent—either 2-mercaptoethanol (2-ME) or dithiothreitol (DTT)—should also be present at a concentration between 1 and 10 mM. The total amount of macromolecule is critical, and should be at least 2 mg, preferably greater than 5 mg.

The first protocols describe the concentration of the macromolecular sample (see Basic Protocol 1 for proteins, see Alternate Protocol 1 for nucleic acids, and see Alternate Protocol 2 for macromolecular complexes). The preparation and assessment of crystallization trials is then described (see Basic Protocol 2), along with a protocol for determining whether the crystals obtained are composed of macromolecule or salt (see Support Protocols 1 and 2). Finally, the optimization of crystallization conditions is presented (see Basic Protocol 3 and Alternate Protocol 3).

PREPARATION OF PROTEIN FOR CRYSTALLIZATION

BASIC PROTOCOL 1

Before a protein can be crystallized it needs to be concentrated to at least 5 mg/ml. An ideal concentration is 20 mg/ml; however, somewhere between 5 and 20 mg/ml is usually suitable.

Materials

Soluble protein sample
Centriprep and Centricon microconcentrators (Amicon)
Dynamic light scattering instrument (optional, but preferable; Protein Solutions model 801 or comparable)
1.5-ml screw-cap microcentrifuge tubes

Additional reagents and equipment for dynamic light scattering (*UNIT 7.8*)

1. Place 15 ml of soluble protein sample in a Centriprep microconcentrator that concentrates 15 ml to ~500 µl, and centrifuge according to manufacturer's instructions.

2. Transfer the sample to a Centricon microconcentrator that concentrates 2 ml to ~50 µl, and continue centrifugation according to manufacturer's instructions. Monitor concentration C (in M) of an appropriately diluted sample ($0.1 \leq A_{280} \leq 1.0$) by determining A_{280} with a UV spectrophotometer and converting with the equation C

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$= A_{280}/\varepsilon l$, where l is the path length (cm), and ε (the molar absorption coefficient in $M^{-1}cm^{-1}$) = (5600 × #Trp) + (1420 × #Tyr) + (197 × #Phe).

For proteins that do not contain Trp or Tyr residues, a colorimetric assay such as the Bradford assay (UNIT 3.4) can be used.

3. Continue to concentrate the protein until it is at ≥5 mg/ml. If this is not attainable, concentrate the protein until visible precipitate is observed.

   If concentration of the protein sample leads to formation of precipitate, the remaining soluble portion should be isolated by centrifugation. Reconcentration of the soluble portion should then be tried under different salt concentrations, pHs, or concentrations of other appropriate additives.

4. If a dynamic light scattering instrument is available, check the protein solution for monodispersity (*UNIT 7.8*).

   Generally, proteins that are monodisperse at high concentrations (typically a polydispersity value ≤15% of the Stokes radius) have a strong tendency to crystallize, and those that are polydisperse have an equally strong tendency not to crystallize (Ferre-D'Amare and Burley, 1994). However, there are exceptions to this rule, and crystallization attempts should not be abandoned solely because of poor dynamic light scattering measurements.

5. Divide into 20- to 50-µl aliquots in 1.5-ml screw-cap microcentrifuge tubes, and flash freeze in liquid nitrogen. Store frozen (preferably at –70°C) until use for crystallization (up to 1 month in most cases).

   If at all possible, subject part of the concentrated protein sample to crystallization trials (see Basic Protocol 2) prior to freezing, as some proteins do not respond well to freeze-thawing. If there is an activity assay available for the protein, it is recommended that the effect of freeze-thawing on this activity be established before freezing the protein sample. If the protein responds adversely, try to freeze the protein sample in the presence of a small amount of glycerol (5% to 15%) or another cryoprotectant. In such cases, it is usually necessary to remove the cryoprotectant (preferably with a microconcentrator) prior to crystallization.

ALTERNATE PROTOCOL 1

PREPARATION OF NUCLEIC ACID FRAGMENTS FOR CRYSTALLIZATION

Before performing this protocol, the oligonucleotide(s) of interest should be purified (preferably by reverse phase HPLC; *UNIT 11.6*), and is usually in dilute solution (i.e., ≤1 mg/ml). The total amount of nucleic acid should be at least 0.5 mg, preferably greater than 1.5 mg. This usually requires solid-phase synthesis at a phosphoramidite scale of 1 µmole or greater.

Materials

DNA sample, purified
10 mM sodium cacodylate (pH 6.8)/50 mM NaCl

Dialysis tubing (Spectra/Por 7, MWCO 2000; Spectrum)
50-ml polypropylene centrifuge tubes (Falcon or Corning)
500-ml lyophilization vessel
1.5-ml screw-cap microcentrifuge tubes

Additional reagents and equipment for dialysis (*UNIT 4.4 & APPENDIX 3B*)

1. Transfer a volume of purified nucleic acid sample that contains ≥0.5 mg oligonucleotide (preferably ≥1.5 mg) to dialysis tubing and dialyze against a total of 200 volumes of water for at least 4 hr at 4°C.
2. Transfer dialyzed material to a 50-ml polypropylene centrifuge tube, cap, and shell freeze the sample (i.e., freeze it onto the sides of the tube) by immersing in crushed dry ice.

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3. Punch holes in the tube cap using a syringe needle, place the tube in a 500-ml lyophilization vessel, and lyophilize the sample to dryness.
4. Redissolve the lyophilized material with 1 ml water and transfer to a 1.5-ml screw-cap microcentrifuge tube. Refreeze the oligonucleotide solution on crushed dry ice, punch small holes in the top of the microcentrifuge tube using a syringe needle, place the tube in a 500-ml lyophilization vessel, and lyophilize to dryness.
5. Dissolve the dried oligonucleotide in 30 µl of 10 mM sodium cacodylate (pH 6.8)/50 mM NaCl.
6. Determine the nucleic acid concentration C (in M) by measuring A_{260} and converting with the equation $C = A_{260}/\varepsilon l$, where l is the path length (cm), and ε (the molar absorption coefficient in M^{-1}) = (15,200 × #dATP) + (9,300 × #dCTP) + (13,700 × #dGTP) + (9,600 × #dTTP) + (9,600 × #dUTP).

For an oligonucleotide that has been synthesized on a 1-µmol phosphoramidate scale, this procedure should yield an oligonucleotide concentration of ~0.8 to 1.5 mM from a minimum of 0.5 mg starting material (step 1).

Form duplexes (optional)

7. Mix complementary strands in stoichiometric molar amounts in a 1.5-ml screw-cap microcentrifuge tube. Tightly seal the microcentrifuge tube.
8. Place the tube in a water bath (i.e., in a beaker) and heat the water bath to 75°C on a hot plate for 5 min.
9. Remove the water bath from the hot plate and cool to 4°C over ~2 hr by allowing the water bath to come to room temperature gradually, then slowly adding ice to the water bath until the bath temperature reaches 10°C, and storing the renatured duplex at 4°C until use for crystallization (up to 2 weeks).

For prolonged storage (greater than 2 weeks), store the sample frozen and renature again prior to use.

PREPARATION OF MACROMOLECULAR COMPLEXES FOR CRYSTALLIZATION

ALTERNATE PROTOCOL 2

There are two types of macromolecular complexes that are generally prepared for crystallization: protein–nucleic acid complexes and protein-protein complexes. For protein–nucleic acid complexes, the concentrated macromolcules (see Basic Protocol 1 and Alternate Protocol 1) are mixed in near stoichiometric amounts. It's been found empirically that it is best to use a slight excess of nucleic acid over protein (~20%; Aggarwal, 1990). In general, one should aim for 0.8 mM of complex.

Some protein–nucleic acid complexes form precipitate when mixed at the high concentrations required for crystallization. There are two common solutions to this problem: (1) lower the concentration of complex by 2- to 3-fold, or (2) add monovalent or divalent metal ions at increasing concentrations. Generally, one should first try adding monovalent ions at a concentration of 25 mM, and increase slowly to a maximum of 200 mM, if the precipitate persists. For divalent ions, one should start at 10 mM and go up to a maximum of 75 mM. NaCl is the most popular monovalent ion, and $MgCl_2$ and $CaCl_2$ are the most commonly used divalent ions. Although divalent ions are often effective in solubilizing some protein–nucleic acid complexes, they should only be used as a last resort, because they may give rise to salt crystals (this typically occurs under crystallization conditions that contain sulfate or phosphate counterions), which may be mistaken for macromolecular crystals (to determine if this is the case, see Support Protocols 1 and 2).

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Homogeneous protein-protein complexes are generally prepared by isolating the desired complex by gel filtration prior to concentration for crystallization. Multiprotein–nucleic acid complexes can be prepared either by mixing the preformed concentrated protein complex with the concentrated nucleic acid sample in appropriate stoichiometric amounts, or by isolating the multiprotein–nucleic acid complex by gel filtration prior to concentration for crystallization.

BASIC PROTOCOL 2

PREPARATION AND ANALYSIS OF CRYSTALLIZATION TRIALS

UNIT 17.3 discusses parameters that effect the crystallization of soluble macromolecules. Overall, the most important parameter in obtaining well-ordered macromolecular crystals is the actual macromolecular sample (see Critical Parameters and Troubleshooting). Additionally, salt, pH, and the precipitating agent all play prominent roles in the crystallization of macromolecules. In theory, there are an infinite number of combinations of these parameters (McPherson, 1982). In practice, however, a set of 50 conditions that have been empirically derived from previously crystallized macromolecules, referred to as sparse matrix sampling conditions, have been very successful in crystallizing a wide range of new macromolecules (Jancarik and Kim, 1991). Typically, each crystallization condition contains a precipitant, a salt, and a buffer. Hampton Research (*http://www.hampton-research.com*) provides a number of different premixed crystallization screens that are optimized for the crystallization of specific macromolecular species. As an example, the components of Crystal Screen 1, used to crystallize proteins, are summarized in Table 17.4.1. A summary of the different screens and their applications is given in Table 17.4.2. Provided that the macromolecular sample is crystallizable, these screens will generally yield (if not diffraction-quality crystals themselves) directions in which to proceed in order to obtain suitable crystals for structural studies.

Although each of the premixed solutions available through Hampton Research can be prepared from scratch, it is convenient, at least initially, to obtain the premixed solutions. Any reagents that are not obtained from Hampton Research should generally be of the highest-quality grade. This is especially true for PEG. This protocol focuses only on screens that are used most often for crystallizing soluble proteins, nucleic acids, and

Table 17.4.1 Crystallization Matrix Components Used in Crystal Screen 1 (Hampton Research) to Crystallize Proteins

Precipitant	Salt	Buffer
Ammonium phosphate	Ammonium acetate	Imidazole, pH 6.5
Ammonium sulfate	Ammonium sulfate	Sodium acetate, pH 4.6
Lithium sulfate	Calcium acetate	Sodium citrate, pH 5.6
Magnesium formate	Calcium chloride	Sodium cacodylate, pH 6.5
MPD[a]	Lithium sulfate	Sodium HEPES, pH 7.5
PEG[b] (400, 1500, 4000, 8000)	Magnesium acetate	Tris·Cl, pH 8.5
Potassium phosphate	Magnesium chloride	
Potassium tartrate	Sodium acetate	
2-Propanol	Sodium citrate	
Sodium acetate	Zinc acetate	
Sodium citrate		
Sodium formate		
Sodium phosphate		
Sodium tartrate		

[a]MPD, 2-methyl-2,4-pentanediol.
[b]PEG, polyethylene glycol.

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Table 17.4.2 Crystallization Screens Available from Hampton Research

Screens for proteins and peptides	Screens for membrane proteins	Screens for nucleic acids and protein–nucleic acid complexes	Optimization screens
Crystal Screen 1[a]	MembFac	Natrix[a]	Additive Screening Kits
Crystal Screen 2[a]		Nucleic Acid Miniscreen	Detergent Screening Kit
Low-Ionic-Strength Screen			
Grid Screen Ammonium Sulfate			
Grid Screen PEG[b] 6000			
Grid Screen MPD[c]			
Grid Screen Sodium Chloride			
Grid Screen PEG/LiCl[b]			

[a]Most frequently used screens.
[b]PEG, polyethylene glycol.
[c]MPD, 2-methyl-2,4-pentanediol.

macromolecular complexes. Grid screens that titrate particular precipitating agents against pH are also provided by Hampton Research. These screens are not used as often and will not be discussed here. Hampton Research also provides many of the supplies required for macromolecular crystallization (see below).

There are several different crystallization techniques that are used for macromolecules (Weber, 1997). Only the one most commonly used, the vapor-diffusion technique using hanging drops (Fig. 17.4.1), is discussed here. Steps 1 to 9 should be carried out both at room temperature and 4°C. Both the sparse crystallization screen (Crystal Screen 1) and the extended sparse crystallization screen (Crystal Screen 2) should be employed for optimal results with proteins, although at a minimum the sparse matrix screen can be used alone. The nucleic acid sparse matrix (Natrix) should be used for nucleic acids or protein–nucleic acid complexes. Crystallization droplets will typically show one of three results: clear drops, crystals, or precipitate/phase separation (see Anticipated Results).

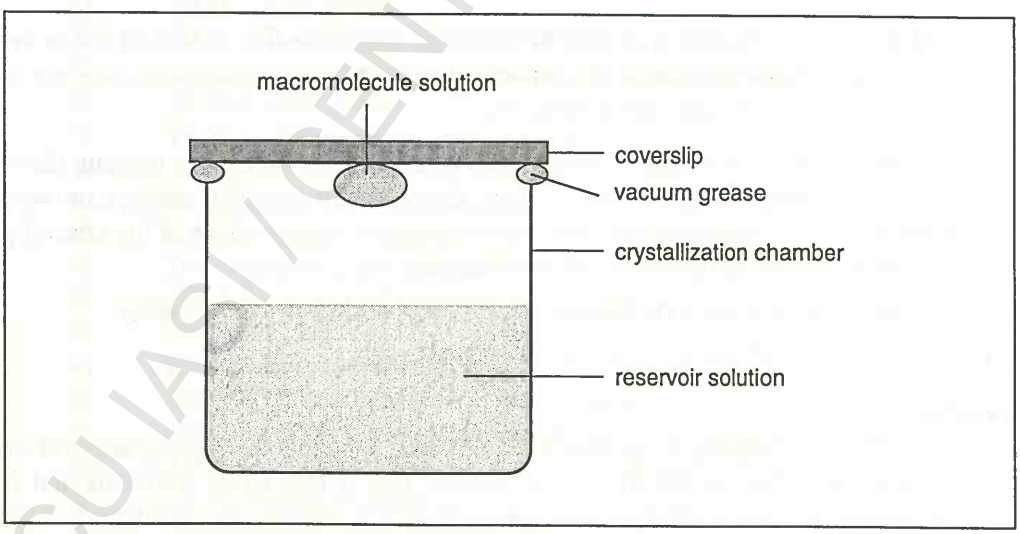

Figure 17.4.1 Typical vapor-diffusion crystallization setup. Components of a typical hanging-drop vapor-diffusion well are schematized.

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Materials

Vacuum grease (Hampton Research)
Crystallization screening kit(s) (Hampton Research): Crystal Screen 1 (for proteins and multiprotein complexes; Table 17.4.3), Crystal Screen 2 (optional; use in addition to Crystal Screen 1 for proteins and multiprotein complexes), or Natrix (for nucleic acids and protein–nucleic acid complexes; Table 17.4.4)
Concentrated macromolecule sample: protein (see Basic Protocol 1), nucleic acid (see Alternate Protocol 1), or macromolecular complex (see Alternate Protocol 2)

10-ml polypropylene syringe
24-well crystallization trays (VDX Plate, Hampton Research)
22-mm^2 plastic coverslips (Hampton Research)
Self-closing tweezers
Microscope [≥40× magnification; Leica MS5, Zeiss STEMI SV8, Nikon SMZ-10, or comparable model, with light source and viewing platform (at least 3 × 4–in.), preferably with polarizer]

Prepare crystallization trays

1. Fill a 10-ml polypropylene syringe with vacuum grease.

   Preparation and crystallization (steps 1 to 9) should be done both at 4°C and at room temperature (±3°C).

2. Apply a uniform layer of vacuum grease to 50 rims of three 24-well crystallization trays using the 10-ml syringe without a needle (i.e., to two entire plates plus two additional wells from a third plate). Do not leave any gaps in the grease. Label the 50 wells, numbering across.

3. Mix the solutions (reservoir mixes) in the appropriate crystallization screening kit according to manufacturer's instructions.

4. Add 500 µl of each reservoir mix to a separate well, using a new pipet tip for each transfer.

Set up hanging drops

5. Place 1 µl concentrated macromolecule sample onto the center of a 22-mm^2 plastic coverslip. Keep stock macromolecule sample on ice.

6. With a new tip, withdraw 1 µl from the reservoir mix in the first well. Add this to the 1-µl sample aliquot and pipet up and down several times to mix, taking care not to introduce air bubbles into the droplet.

7. Flip the coverslip over with self-closing tweezers so that the drop is hanging down from the bottom of the coverslip. Place the coverslip over the appropriate well solution. To seal the coverslip-well interface, gently tap the edges of the coverslip into the grease using the bottom of the tweezers.

   Figure 17.4.1 illustrates the components of a completed vapor-diffusion setup.

8. Repeat steps 5 to 7 for the rest of the well solutions.

Incubate

9. After all of the hanging drops have been prepared, tape together the top and bottom of each tray. Transfer the trays to a location that is free from vibrations and is maintained at a fairly constant temperature (±3°C). Leave the trays undisturbed for at least 2 days.

   In general, crystallization trays should not be discarded for at least 6 months. Some proteins have been known to undergo time-dependent modifications that promote crystallization.

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Table 17.4.3 Components of Crystal Screen 1 (Hampton Research) for Soluble Proteins[a]

Condition	Formulation[b]
1	30% MPD, 0.1 M sodium acetate, pH 4.6, 0.02 M calcium chloride
2	0.4 M potassium/sodium tartrate
3	0.4 M ammonium phosphate
4	2.0 M ammonium sulfate, 0.1 M Tris·Cl, pH 8.5
5	30% MPD, 0.1 M sodium HEPES, pH 7.5, 0.2 M sodium citrate
6	30% PEG 4000, 0.1 Tris·Cl, pH 8.5, 0.2 M magnesium chloride
7	1.4 M sodium acetate, 0.1 M sodium cacodylate, pH 6.5
8	30% 2-propanol, 0.1 M sodium cacodylate, pH 6.5, 0.2 M sodium citrate
9	30% PEG 4000, 0.1 M sodium citrate, pH 5.6, 0.2 M ammonium acetate
10	30% PEG 4000, 0.1 M sodium acetate, pH 4.6, 0.2 M ammonium acetate
11	1.0 M ammonium phosphate, 0.1 M sodium citrate, pH 5.6
12	30% 2-propanol, 0.1 M sodium HEPES, pH 7.5, 0.2 M magnesium chloride
13	30% PEG 400, 0.1 M Tris·Cl, pH 8.5, 0.2 M sodium citrate
14	28% PEG 400, 0.1 M sodium HEPES, pH 7.5, 0.2 M calcium chloride
15	30% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, 0.2 M ammonium sulfate
16	1.5 M lithium sulfate, 0.1 M sodium HEPES, pH 7.5
17	30% PEG 4000, 0.1 M Tris·Cl pH 8.5, 0.2 M lithium sulfate
18	20% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, 0.2 M magnesium acetate
19	30% 2-propanol, 0.1 M Tris·Cl, pH 8.5, 0.2 M ammonium acetate
20	25% PEG 4000, 0.1 M sodium acetate, pH 4.6, 0.2 M ammonium sulfate
21	30% MPD, 0.1 M sodium cacodylate, pH 6.5, 0.2 M magnesium acetate
22	30% PEG 4000, 0.1 M Tris·Cl, pH 8.5, 0.2 M sodium acetate
23	30% PEG 400, 0.1 M sodium HEPES, pH 7.5, 0.2 M magnesium chloride
24	20% 2-propanol, 0.1 M sodium acetate, pH 4.6, 0.2 M calcium chloride
25	1.0 M sodium acetate, 0.1 M imidazole, pH 6.5
26	30% MPD, 0.1 M sodium citrate, pH 5.6, 0.2 M ammonium acetate
27	20% 2-propanol, 0.1 M sodium HEPES, pH 7.5, 0.2 M sodium citrate
28	30% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, 0.2 M sodium acetate
29	0.8 M potassium/sodium tartrate, 0.1 M sodium HEPES, pH 7.5
30	30% PEG 8000, 0.2 M ammonium sulfate
31	30% PEG 4000, 0.2 M ammonium sulfate
32	2.0 M ammonium sulfate
33	4.0 M sodium formate
34	2.0 M sodium formate, 0.1 M sodium acetate, pH 4.6
35	1.6 M sodium/potassium phosphate, 0.1 M sodium HEPES, pH 7.5
36	8% PEG 8000, 0.1 M Tris·Cl, pH 8.5
37	8% PEG 4000, 0.1 M sodium acetate, pH 4.6
38	1.4 M sodium citrate, 0.1 M sodium HEPES, pH 7.5
39	2% PEG 400, 2.0 M ammonium sulfate, 0.1 M sodium HEPES, pH 7.5
40	20% 2-propanol, 20% PEG 4000, 0.1 M sodium citrate, pH 5.6
41	10% 2-propanol, 20% PEG 4000, 0.1 M sodium HEPES, pH 7.5
42	20% PEG 8000, 0.05 M potassium phosphate
43	30% PEG 1500
44	0.2 M magnesium formate
45	18% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, 0.2 M zinc acetate
46	18% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, 0.2 M calcium acetate
47	2.0 M ammonium sulfate, 0.1 M sodium acetate, pH 4.6
48	2.0 M ammonium phosphate, 0.1 M Tris·Cl, pH 8.5
49	2% PEG 8000, 1.0 M lithium sulfate
50	15% PEG 8000, 0.5 M lithium sulfate

[a] Jancarik and Kim (1991).

[b] MPD, 2-methyl-2,4-pentanediol; PEG, polyethylene glycol.

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Table 17.4.4 Components of Natrix (Hampton Research) for Nucleic Acid and Protein–Nucleic Acid Complexes[a]

Condition	Formulation[b]
1	0.01 M magnesium chloride, 0.05 M MES, pH 5.6, 2.0 M lithium sulfate
2	0.01 M magnesium acetate, 0.05 M MES, pH 5.6, 2.5 M ammonium sulfate
3	0.1 M magnesium acetate, 0.05 M MES, pH 5.6, 20% MPD
4	0.2 M KCl, 0.01 M magnesium sulfate, 0.05 M MES, pH 5.6, 10% PEG 400
5	0.2 M KCl, 0.01 M magnesium chloride, 0.05 M MES, pH 5.6, 5% PEG 8000
6	0.1 M ammonium sulfate, 0.01 M magnesium chloride, 0.05 M MES, pH 5.6, 20% PEG 8000
7	0.02 M magnesium chloride, 0.05 M MES, pH 6.0, 15% isopropanol
8	0.005 M magnesium sulfate, 0.1 M ammonium acetate, 0.05 M MES, pH 6.0, 0.6 M NaCl
9	0.1 M KCl, 0.01 M magnesium chloride, 0.05 M MES, pH 6.0, 10% PEG 400
10	0.005 M magnesium sulfate, 0.05 M MES, pH 6.0, 5% PEG 4000
11	0.01 M magnesium chloride, 0.05 M sodium cacodylate, pH 6.0, 1.0 M lithium sulfate
12	0.01 M magnesium sulfate, 0.05 M sodium cacodylate, pH 6.0, 1.8 M lithium sulfate
13	0.015 M magnesium acetate, 0.05 M sodium cacodylate, pH 6.0, 1.7 M ammonium sulfate
14	0.1 M KCl, 0.025 M magnesium chloride, 0.05 M sodium cacodylate, pH 6.0, 15% isopropanol
15	0.04 M magnesium chloride, 0.05 M sodium cacodylate, pH 6.0, 5% MPD
16	0.04 M magnesium acetate, 0.05 M sodium cacodylate, pH 6.0, 30% MPD
17	0.2 M KCl, 0.01 M calcium chloride, 0.05 M sodium cacodylate, pH 6.0, 10% PEG 4000
18	0.01 M magnesium acetate, 0.05 M sodium cacodylate, pH 6.5, 1.3 M lithium sulfate
19	0.01 M magnesium sulfate, 0.05 M sodium cacodylate, pH 6.5, 2.0 M ammonium sulfate
20	0.1 M ammonium acetate, 0.015 M magnesium acetate, 0.05 M sodium cacodylate, pH 6.5, 10% isopropanol
21	0.2 M KCl, 0.005 M magnesium chloride, 0.05 M sodium cacodylate, pH 6.5, 10% 1,6-hexanediol
22	0.08 M magnesium acetate, 0.05 M sodium cacodylate, pH 6.5, 15% PEG 400
23	0.2 M KCl, 0.01 M magnesium chloride, 0.05 M sodium cacodylate, pH 6.5, 10% PEG 4000
24	0.2 M ammonium acetate, 0.01 M calcium chloride, 0.05 M sodium cacodylate, pH 6.5, 10% PEG 4000
25	0.08 M magnesium acetate, 0.05 M sodium cacodylate, pH 6.5, 30% PEG 4000
26	0.2 M KCl, 0.1 M magnesium acetate, 0.05 M sodium cacodylate, pH 6.5, 10% PEG 8000
27	0.2 M ammonium acetate, 0.01 M magnesium acetate, 0.05 M sodium cacodylate, pH 6.5, 30% PEG 8000
28	0.05 M magnesium sulfate, 0.05 M sodium HEPES, pH 7.0, 1.6 M lithium sulfate
29	0.01 M magnesium chloride, 0.05 M sodium HEPES, pH 7.0, 4.0 M lithium chloride
30	0.01 M magnesium chloride, 0.05 M sodium HEPES, pH 7.0, 1.6 M ammonium sulfate

continued

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Table 17.4.4 Components of Natrix (Hampton Research) for Nucleic Acid and Protein–Nucleic Acid Complexes[a], continued

Condition	Formulation[b]
31	0.005 M magnesium chloride, 0.05 M sodium HEPES, pH 7.0, 25% PEG monomethyl ether 550
32	0.2 M KCl, 0.01 M magnesium chloride, 0.05 M sodium HEPES, pH 7.0, 20% 1,6-hexanediol
33	0.2 M ammonium chloride, 0.01 M magnesium chloride, 0.05 M sodium HEPES, pH 7.0, 30% 1,6-hexanediol
34	0.1 M KCl, 0.005 M magnesium sulfate, 0.05 M sodium HEPES, pH 7.0, 15% MPD
35	0.1 M KCl, 0.01 M magnesium chloride, 0.05 M sodium HEPES, pH 7.0, 5% PEG 400
36	0.1 M KCl, 0.01 M calcium chloride, 0.05 M sodium HEPES, pH 7.0, 10% PEG 400
37	0.2 M KCl, 0.025 M magnesium sulfate, 0.05 M sodium HEPES, pH 7.0, 20% PEG 200
38	0.2 M ammonium acetate, 0.15 M magnesium acetate, 0.05 M sodium HEPES, pH 7.0, 5% PEG 4000
39	0.1 M ammonium acetate, 0.02 M magnesium chloride, 0.05 M sodium HEPES, pH 7.0, 5% PEG 8000
40	0.01 M magnesium chloride, 0.05 M Tris·Cl, pH 7.5, 1.6 M ammonium sulfate
41	0.1 M KCl, 0.015 M magnesium chloride, 0.05 M Tris·Cl, pH 7.5, 10% PEG monomethyl ether 550
42	0.01 M magnesium chloride, 0.05 M Tris·Cl, pH 7.5, 5% isopropanol
43	0.01 M magnesium chloride, 0.05 M ammonium acetate, 0.05 M Tris·Cl, pH 7.5, 10% MPD
44	0.2 M KCl, 0.05 M magnesium chloride, 0.05 M Tris·Cl, pH 7.5, 10% PEG 4000
45	0.025 M magnesium sulfate, 0.05 M Tris·Cl, pH 8.5, 1.8 M ammonium sulfate
46	0.005 M magnesium sulfate, 0.05 M Tris·Cl, pH 8.5, 35% 1,6-hexanediol
47	0.1 M KCl, 0.01 M magnesium chloride, 0.05 M Tris·Cl, pH 8.5, 30% PEG 400
48	0.01 M calcium chloride, 0.2 M ammonium chloride, 0.05 M Tris·Cl, pH 8.5, 30% PEG 4000

[a]Scott et al. (1995).

[b]MES, 2-(*N*-morpholino)ethanesulfonic acid; MPD, 2-methyl-2,4-pentanediol; PEG, polyethylene glycol.

Analyze results

10. Use a microscope (preferably with polarizer) to view the crystallization drops. Note whether the drops are clear (0), show crystals (C), or show precipitate or phase separation (P).

11. For those drops that produce crystals, note the crystallization conditions and see if there is a common parameter (i.e., precipitant, pH, or ionic strength). Also note the crystal size, shape, and ability to polarize light.

    In general, large (≥0.4 mm^3) crystals that have sharp edges and that polarize light are preferable.

12. Continue to observe the crystallization trials every 2 days until no further change is noted in the drops.

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ESTABLISHING WHETHER CRYSTALS ARE MACROMOLECULE OR SALT USING A CRYOLOOP

Often one obtains crystals that are salt rather than macromolecule. This notoriously occurs when the crystallization conditions contain divalent ions such as magnesium or calcium with phosphate or sulfate counterions. If crystals are suspected to be salt, prepare a control crystallization trial in which everything but the macromolecule is included. In preparing this control, substitute the macromolecule sample with the storage buffer that is identical to that used for the macromolecule in the drops. If this test does not show crystals one can analyze a washed crystal directly by SDS-PAGE (*UNIT 10.1*). The procedure for preparing an SDS-PAGE sample from a crystal is described here. A crystal that is at least 0.4 mm^3 is usually large enough to wash with a cryoloop and dissolve for visualization by SDS-PAGE and silver staining (*UNIT 10.5*; both proteins and nucleic acids are stained with silver reagent). The procedure involves extensively washing the crystal surface, so that it is free of macromolecule from the hanging drop, before dissolving the crystal for gel analysis. For microcrystals, see Support Protocol 2.

Materials

Crystallization tray(s) containing 0.4-mm^3 (or larger) crystal(s) (see Basic Protocol 2)
Macromolecule storage solution (solution in which the macromolecule is soluble)
Purified macromolecule

Microscope [≥40× magnification; Leica MS5, Zeiss STEMI SV8, Nikon SMZ-10, or comparable model, with light source and viewing platform (at least 3 × 4–in.), preferably with polarizer]
Cryoloops (Hampton Research)
Plastic petri dish

Additional reagents and equipment for SDS-PAGE (*UNIT 10.1*) and silver staining (*UNIT 10.5*)

1. With a microscope nearby, invert the coverslip harboring 0.4-mm^3 crystals and place on a flat surface. Pipet 100 µl of the respective reservoir solution directly to the drop.
2. While viewing the drop under the microscope, use a cryoloop to mix the solution in the drop, and gently move the crystal to make sure it is detached from the coverslip surface.
3. Pipet three separate 100-µl drops of reservoir solution into a plastic petri dish.
4. Place the cryoloop under the crystal such that the crystal is hanging half inside and half outside the circle, and then lift the loop swiftly upward. Transfer the crystal to the first 100-µl drop, shake slightly to pry the crystal off the loop, and mix the solution slightly.

   When the crystal is "lassoed" in this manner, it is held in the loop by the surface tension of the liquid that is attached to the rim of the loop.
5. Transfer the crystal to the second drop, mix slightly by swirling the loop in the reservoir solution, and finally transfer the crystal to the third drop.
6. Use the cryoloop to transfer the crystal to a microcentrifuge tube containing 5 µl of macromolecule storage solution, and shake the cryoloop to release the crystal. Vortex the solution to fully dissolve the crystal.
7. For a negative control, transfer 5 µl from the third washing drop to a microcentrifuge tube containing 5 µl of the macromolecule storage solution. For a positive control,

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add 1 µg purified macromolecule in a final volume of 5 µl macromolecule storage solution to another microcentrifuge tube.

This negative control ensures that all of the free macromolecule in solution has been washed from the crystal surface.

8. Add 5 µl 2× SDS sample buffer (*UNIT 10.1*) to each tube and analyze the samples by SDS-PAGE (*UNIT 10.1*). Visualize macromolecule(s) by silver staining the gel (*UNIT 10.5*).

ESTABLISHING WHETHER MICROCRYSTALS ARE MACROMOLECULE OR SALT BY FILTRATION

SUPPORT PROTOCOL 2

Often one obtains a shower of small microcrystals that are difficult to manipulate by the procedure outlined in Support Protocol 1. In such cases, the procedure outlined below can be used to establish whether the microcrystals contain macromolecules.

Additional Materials (also see Support Protocol 1)

Centricon-100 microconcentrator (MWCO 100; Amicon)
1.5-ml screw-cap microcentrifuge tube
500-ml lyophilization vessel

1. Invert the coverslip harboring the crystals and use a 10-µl pipettor to transfer the drop to a Centricon-100 microconcentrator.
2. Wash the coverslip surface with 50 µl of the reservoir solution used to obtain the crystals. Wash the coverslip by pipetting up and down several times with a 200-µl pipettor. Use 200-µl pipettor to transfer this wash solution to the same microconcentrator containing the drop.
3. Add an additional 500 µl reservoir solution to the microconcentrator and concentrate the entire sample by centrifugation to dead volume, per manufacturer's instructions.
4. Add 2 ml of additional reservoir solution to the microconcentrator and reconcentrate by centrifugation to dead volume.
5. Add 100 µl macromolecule storgae solution to the microconcentrator and mix by pipetting up and down several times with a 200-µl pipettor.
6. Invert the microconcentrator, and centrifuge to recover sample, which should now contain the dissolved crystals.
7. Transfer the sample to a 1.5-ml screw-cap microcentrifuge tube, punch holes in the top using a syringe needle, and freeze over crushed dry ice. Place in a 500-ml lyophilization vessel and lyophilize the sample to dryness.
8. Redissolve the lyophilized sample with 5 µl macromolecule storage solution and 5 µl of 2× SDS-PAGE sample buffer (*UNIT 10.1*). Prepare controls (see Support Protocol 1, step 7), and analyze by SDS-PAGE (*UNIT 10.1*) Visualize the sample by silver staining the gel (*UNIT 10.5*).

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OPTIMIZATION OF CRYSTALLIZATION PARAMETERS

Although crystals that are obtained from sparse matrix screens are sometimes already suitable for structural studies, more often the crystals need to be optimized prior to crystallographic analysis. Common problems include crystals that are too small, too thin, clustered, or intergrown. In these cases, the optimization of precipitant concentration, pH, and ionic strength often improve crystal quality. There are different ways of optimizing crystallization parameters. The procedure generally used in the author's laboratory is presented here. Figure 17.4.2 illustrates a typical optimization procedure, which is discussed further in annotations to the steps below.

Materials

Microscope [≥40× magnification; Leica MS5, Zeiss STEMI SV8, Nikon SMZ-10, or comparable model) with light source and viewing platform (at least 3 × 4–in.), preferably with polarizer]

Additional reagents and equipment for preparation of crystallization trials (see Basic Protocol 2)

1. For each crystallization condition that yields crystals, prepare a crystallization tray (see Basic Protocol 2, steps 1 and 2) that contains an initial one-dimensional grid to optimize the concentration of precipitant. Design precipitant concentrations that range in 10% and 15% increments from zero to ~20% higher than that used in the screen condition.

   As an example (Fig. 17.4.2A), protein crystallization trials (see Basic Protocol 2) using Crystal Screen 1 (Table 17.4.3) yielded a shower of nicely shaped but small crystals under condition 9, which contains 30% polyethylene glycol (PEG) 4000, 0.2 M ammonium acetate, 0.1 M sodium citrate, pH 5.6. This condition was used as a starting point for further optimization. Starting protein concentration in the crystallization drop is 10 mg/ml. Figure 17.4.2A shows the first grid screen that was prepared to optimize the precipitant concentration.

2. Prepare reservoirs by adding the appropriate amounts of stock precipitant, buffer, and salt directly to the well and diluting with water to a final volume of 1 ml. Mix by pipetting up and down several times with a 1-ml pipettor.

3. In each well, mix 1 µl macromolecule with 1 µl reservoir solution and equilibrate several days over the appropriate reservoir as described in Basic Protocol 2 (steps 5 to 9).

   Trays should be incubated at the same temperature used to obtain the initial crystals.

4. Observe crystallization trays under a microscope, and note the conditions that yield the best crystals (i.e., those with the sharpest edges, largest size, and most invariant form). Continue to observe crystallization trays for at least 1 week.

   After 1 week in the example, it was determined that crystals prepared from reservoir 3 were bigger and larger than those prepared from reservoirs 4 and 5. The condition for this reservoir (15% PEG 4000, 0.2 M ammonium acetate, 0.1 M sodium citrate, pH 5.6) was used as a starting point to optimize pH and ionic strength.

5. Prepare a two-dimensional grid to optimize pH and ionic strength conditions as a function of precipitant concentration (e.g., Fig. 17.4.2B). Vary the precipitant concentration more narrowly, based on the results of steps 1 to 4. Vary the ionic strength (by 20% to 25% increments) and pH (by 1-pH-unit increments) both below and above the values used in the initial screen. Set up and observe trials as in steps 2 to 4 above.

   Note that different buffers will have to be used to establish different pH values. Generally, use the buffers that are employed in the sparse matrix screens.

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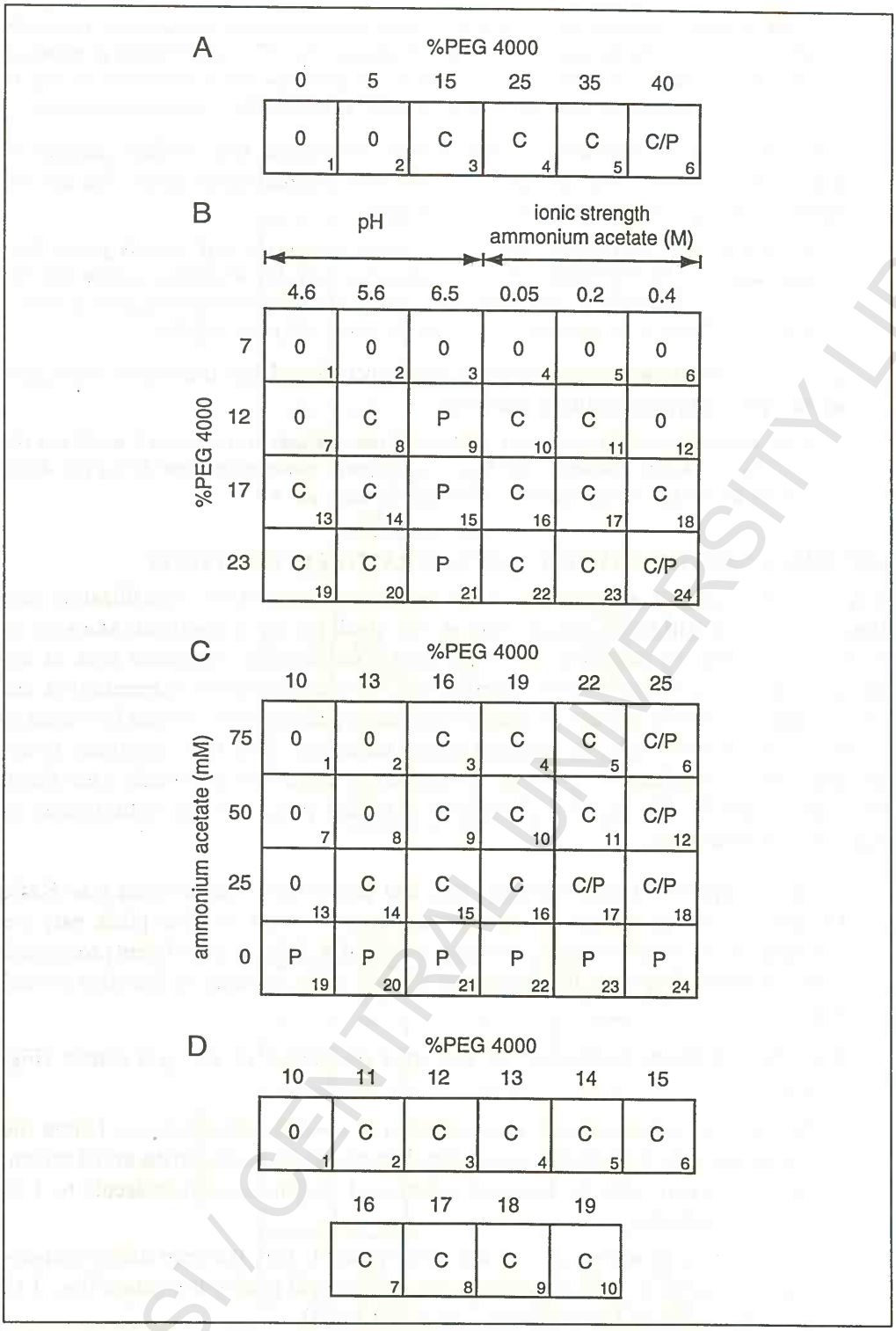

Figure 17.4.2 Optimization of crystallization parameters (see Basic Protocol 3 for discussion). (**A**) One-dimensional grid screen to optimize precipitant conditions, performed at 0.1 M sodium citrate, pH 5.6, and 0.2 M ammonium acetate. (**B**) Two-dimensional expansion of conditions to optimize pH (held at constant ionic strength with 0.2 M ammonium acetate) and ionic strength (held at constant pH with 0.1 M sodium citrate, pH 5.6), as well as small changes in precipitant. pH optimation performed using 0.1 M sodium acetate (ph 4.6), 0.1 M sodium citrate (pH 5.6), 0.1 M sodium cacodylate (pH 6.5). (**C**) Additional two-dimensional grid to optimize smaller changes in ionic strength and precipitant (held at constant pH with 0.1 M sodium acetate, pH 4.6). (**D**) Continued optimization of precipitant (at 0.1 M sodium acetate, pH 4.6, and 35 mM ammonium acetate). Optimal crystallization parameters were identified for reservoirs 2 and 3. Symbols: 0, clear; C, crystal; P, precipitate and/or phase separation.

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In the example, observations made after 1 week determined that crystals from reservoirs 10 and 13 looked the longest and most nicely shaped (Fig. 17.4.2B). Crystals in reservoir 18 looked striated with blunt edges. Therefore, the conditions from reservoirs 10 and 13 were used to further optimize the conditions at pH 4.6 and 0.05 M ammonium acetate.

6. To more finely to optimize crystal growth conditions, use smaller changes of precipitating agent, ionic strength, and pH with 2-dimensional grids. Set up and observe trials as described in steps 2 to 4 above.

   In the example, observations made after 1 week determined that crystals grown from reservoirs 9 (16% PEG 4000, 50 mM ammonium acetate, 0.1 M sodium acetate, pH 4.6) and 14 (13% PEG 4000, 25 mM ammonium acetate, 0.1 M sodium acetate, pH 4.6) looked best (Fig. 17.4.2C); the crystals in reservoir 14 looked marginally better.

7. Use these conditions to prepare the next experimental grid. Use the results of this grid as the optimal crystallization parameters.

   In the example, observation of this grid shows that crystals in reservoirs 2 and 3 are the best (Fig. 17.4.2D). Therefore, the best crystallization parameters were 11% PEG 4000, 35 mM ammonium acetate, and 0.1 M sodium acetate, pH 4.6.

ALTERNATE PROTOCOL 3

OPTIMIZATION OF CRYSTAL EQUILIBRATION CONDITIONS

It is often the case that optimization of the chemical parameters for crystallization (see Basic Protocol 3) still yields crystals that are too small for crystallographic analysis. In such cases, it may be helpful to use a different crystallization procedure such as the sitting-drop method (Weber, 1997). Alternatively, the macromolecule concentration, the ratio of macromolecule solution to reservoir solution, and the drop size can be varied to obtain larger crystals using the vapor-diffusion technique. This latter approach is described here. Crystallization trials are performed as described previously (see Basic Protocols 2 and 3). Figure 17.4.3 illustrates a typical procedure for optimization of equilibration conditions.

1. Using the optimized ionic strength, pH, and precipitant concentration (see Basic Protocol 3), design a set of two-dimensional grids. In each of these grids, vary the precipitant concentration finely along the 4-well axis, using four different precipitant concentrations. Duplicate this parameter in each of the columns in the other crystal trays.

2. Vary three different parameters in the other dimension of the grid screen (Fig. 17.4.3A).

   a. *To decrease macromolecule concentration in constant drop volume:* Dilute the macromolecule 2- and 4-fold relative to the concentration used in the initial screen, prior to mixing with the reservoir solution (1 µl dilute macromolecule to 1 µl reservoir solution).

   b. *To increase drop volume at constant concentration:* Vary the drop size by increasing the volume of both macromolecule solution and reservoir solution (i.e., 1 µl macromolecule to 1 µl reservoir, 2 to 2, and 3 to 3).

   c. *To shift the equilibration process:* Vary the reservoir solution/macromolecule solution ratio (i.e., 1:2, 2:1, 2:3, 3:2) in a constant drop volume (2 µl).

      Using the conditions determined in Basic Protocol 3 (see Fig. 17.4.2D), a screen to optimize crystallization equilibration conditions was prepared (Figs. 17.4.3A).

3. Set up trays as described in Basic Protocol 3 and observe them after several days.

   In the example (Fig. 17.4.3A), observations made after 1 week determined that reservoirs 14, 18, and 31 gave the largest crystals. Thus, the best parameters are 5 mg/ml protein mixed at a 2:3 protein/reservoir ratio in a final drop volume of 6 µl.

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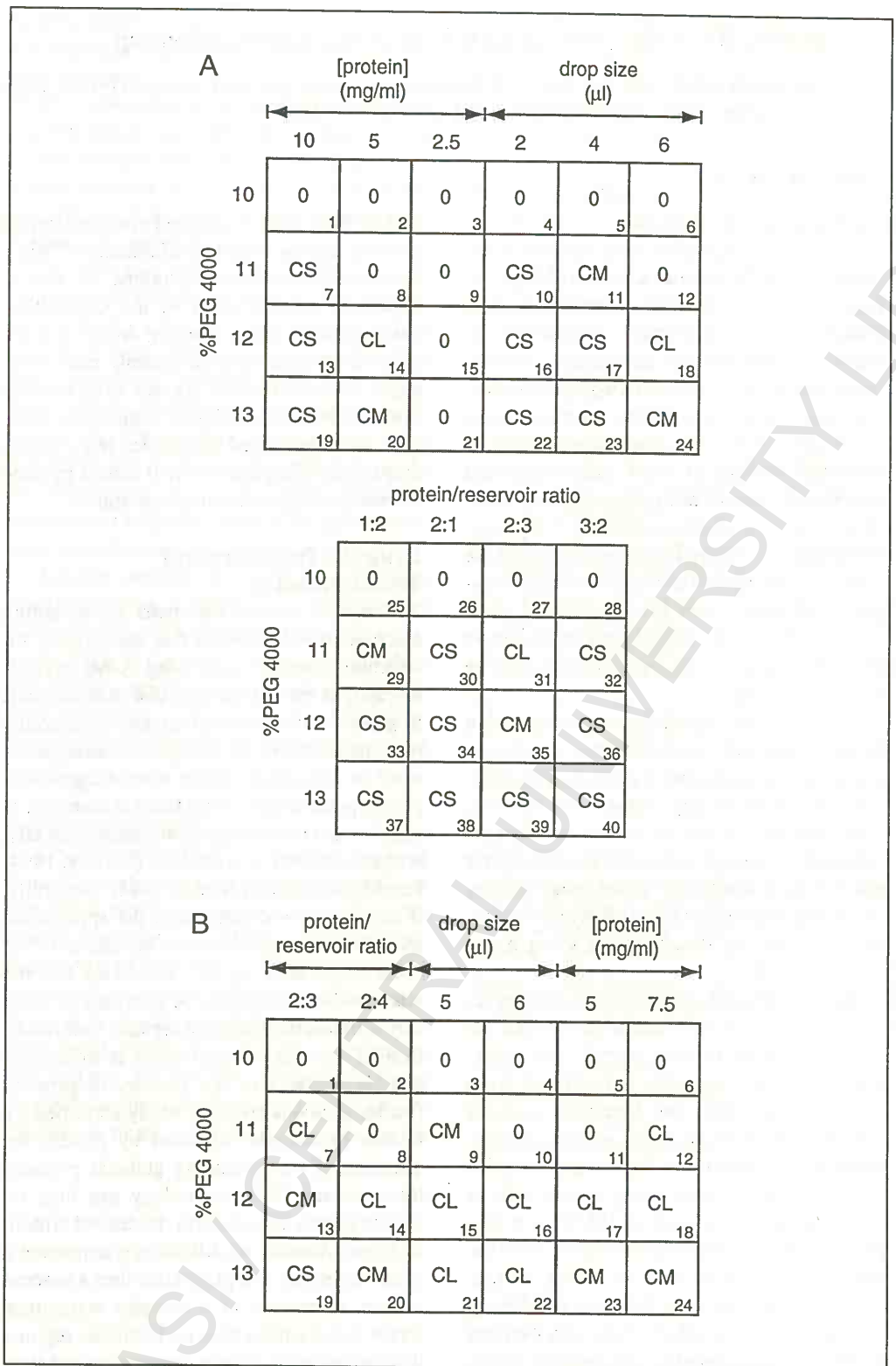

Figure 17.4.3 Optimization of crystal equilibration parameters (see Alternate Protocol 3 for discussion). (**A**) Determination of protein concentration, drop size, and protein solution/reservoir solution ratio (protein/reservoir ratio). (**B**) Optimization of all parameters. All performed at 0.1 M sodium acetate, pH 4.6, and 35 mM ammonium acetate. Protein concentrators are indicated for the crystallization drop (i.e., after 1:1 dilution of protein in reservoir solution), and are shown for the initial trial (10 mg/ml) and for 2- and 4-fold dilutions. Drop size is varied at a constant protein concentration of 10 mg/ml. The protein/reservoir solution ratio is varied using a 20 mg/ml protein stock solution (i.e., the 1:2 and 2:1 dilutions have final protein concentrations of 6.7 and 13.3 mg/ml, respectively). Symbols: 0, clear; CS, small crystals; CM, medium crystals; CL, large crystals.

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4. *Optional:* If necessary, refine drops with an additional optimization step.

In the example, one last round of optimization testing provided several crystals large enough for X-ray crystallographic analysis (Fig. 17.4.3B).

COMMENTARY

Background Information

X-ray crystallography has undergone a renaissance over the last decade. This change has been due to a combination of several factors. Among the most important of these are advances in data collection techniques, computational resources, and crystallographic refinement procedures. In addition, advances in recombinant DNA technology have opened up structural biology to many more types and varieties of proteins and macromolecular complexes. Because of these advances, X-ray crystallography has moved from being a technique that was only applied to available protein samples to one that is open to any biological question. In other words, crystallography no longer drives the questions; biological questions drive the crystallography.

A significant result of this renaissance is that the procedures of crystallography are no longer restricted to a dedicated crystallography laboratory. Crystallography is now a tool used by many laboratories that do not necessarily specialize in crystallographic analysis. In fact, it is quite straightforward for a molecular biology laboratory to prepare crystals that may be suitable for structure determination using X-ray crystallography.

Macromolecular crystallization is the process by which macromolecules are coaxed out of solution in the form of crystals. The induction of crystal formation is facilitated by a precipitating agent that functions to drive equilibration between small macromolecule-containing solution and large reservoir solution. A volatile precipitating agent, such as 2-methyl-2,4-pentanediol (MPD) or isopropanol, transfers by vapor-diffusion from the reservoir to the drop, thus increasing the precipitant concentration in the drop and driving the macromolecule out of solution (in the form of precipitate or crystals). Alternatively, a nonvolatile precipitating agent, such as salt or large polyethylene glycol (PEG), functions to promote the diffusion of water from the drop to the reservoir, causing the precipitant concentration to increase in the drop and thus driving the macromolecule out of solution. A more detailed discussion underlying the theory of crystal formation from a supersaturated macromolecular solution can be found in McPherson (1982). Here we present the technique of macromolecular crystallization by the vapor-diffusion technique using hanging drops. It is the most convenient most commonly used technique, and often yields crystals from suitable macromolecular samples. In some cases, however, more advanced techniques (e.g., sitting drops, microdialysis; *UNIT 17.3*) should be used if these procedures are unsuccessful.

Critical Parameters and Troubleshooting

The most critical parameter for obtaining macromolecular crystals that are suitable for structure determination using X-ray crystallography is the macromolecular sample itself. In general, homogeneous, compact, monodisperse macromolecular samples are straightforward to crystallize, while nonhomogeneous, polydisperse samples with flexible domains, or samples that tend to aggregate nonspecifically, are very difficult to crystallize (D'Arcy, 1994; Ferre-D'Amare and Burley, 1994). Generally, if crystals do not form with the appropriate sparse matrix crystallization screens at either room temperature or 4°C, it is likely that the macromolecule needs to be modified in some way to obtain well-ordered crystals. One modification that can be performed is to remove flexible domains of the protein. In general, flexible domains are most easily identified by limited proteolysis, followed by protein sequencing of the remaining globular product. Recombinant DNA technology can then be used to genetically engineer the desired protein fragment. Another modification is to prepare a macromolecular complex rather then a nascent protein. Formation of a complex sometimes serves to bury otherwise hydrophobic regions that can nucleate protein aggregation and thus hamper crystallization. In this regard, protein-antibody complexes sometimes crystallize more readily then free proteins.

Aside from the macromolecular sample, physiologically relevant cofactors often play major roles in crystallization. For example, enzymes may show entirely different crystal-

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lization behavior when they are bound to various nonhydrolyzable small-molecule or peptide substrates.

The most common problem associated with macromolecular crystallization is that the protein precipitates either upon concentration or in a large majority of crystallization conditions. This often indicates that the protein has a strong tendency to aggregate at high concentrations. If this is the case, one of the two modification strategies described above should be considered. Diluting the macromolecule 2- to 4-fold may also help. Alternatively, crystallization trays may show predominantly clear drops. This indicates that the protein concentration is too low and needs to be raised (between 2- and 4-fold), or that the protein is overly soluble. In the latter case, one might try crystallization screens at or very near the pI of the macromolecule, in order to reduce its charge.

Another common problem in crystallizing macromolecules is that crystals of salt rather than of macromolecule are obtained. To determine if this is the case, set up a control drop containing everything except macromolecule. Salt crystals are commonly obtained with divalent ions (particularly with phosphate or sulfate counterions).

Anticipated Results

Crystallization trials generally show one of three types of results: clear drops, crystals, or precipitate or phase separation. Most often, one obtains a mixture of all of these results depending on the well conditions. If crystals are obtained under several different screen conditions, it is usually straightforward to proceed from small crystals to well-ordered, large crystals suitable for structure analysis by X-ray crystallography. Conditions that result in precipitate or phase separation generally are incompatible with crystal formation. If all, or nearly all, of the drops show precipitate, this is a bad sign and suggests that a modification of the macromolecule is required (see Critical Parameters and Troubleshooting).

Time Considerations

Once a purified macromolecule is on hand, it can usually be concentrated and set up in crystallization trials in a matter of a few days. Assessment and refinement of crystallization parameters can take between a week to several months, depending on the behavior of the macromolecular sample.

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PREPARATION AND HANDLING OF PEPTIDES

18

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CHAPTER 18
Preparation and Handling of Peptides

INTRODUCTION

An important component of the armamentarium of modern protein scientists is the ability to design and construct peptides of almost any sequence. Peptides may be employed for a wide range of applications, including as substrates for kinases, proteases, or glycosidases, or as antigens to produce antisera that may recognize a protein containing that sequence. Peptides also have many biological functions on their own, interacting with receptors to stimulate changes in cellular function, or altering the interactions between cells, for example.

Chemists developed the means to synthesize peptides early in this century (*UNIT 18.1*). The ability to create a molecule that possessed biological activity by organic synthesis is certainly a milestone in our science. For many years, however, peptide synthesis was practiced only by highly skilled and dedicated individuals, and the lengthy time required to obtain even simple peptides limited their use as reagents for biological experiments. Difficulties in purification effectively eliminated the study of complex peptides of more than 5 to 10 amino acids.

Several developments have revolutionized this field, fortunately, and they have brought peptide science into the realm of standard biological techniques available to many scientists. First was the development of solid-phase methods by Bruce Merrifield and subsequent improvements in the reagents available for synthesis that are detailed in *UNIT 18.1*. Second was the design and construction of automated instruments to perform the repetitive steps of the solid-phase methodology, culminating in the 1980s with instruments that provided nearly global accessibility to synthetic peptides of ever increasing length.

Another advance was the optimization of reversed-phase HPLC as a separation method for the rapid purification and analysis of synthetic peptides (*UNIT 11.6*). A future unit in this chapter will focus on medium-to-large scale peptide purification by HPLC methods. In addition, recent advances in mass spectroscopy (Chapter 16) have provided another critical analytical tool to verify the products of synthesis.

A convenient, low-cost method for creating large numbers of related peptides or for assembly of a set of peptides that together span an entire protein to search for the epitope recognized by an antibody that binds to the full-length protein is provided by the synthesis of peptides on a multipin apparatus (*UNIT 18.2*).

Frequently, genomic studies lead to the discovery of an open reading frame (ORF), predicting a protein sequence that might have an activity of interest. One step that could follow would be the generation of an antibody to use as a reagent to identify the protein product of the gene in a sample isolated from the organism under study. A small peptide of 10 to 15 amino acids can frequently be used as an antigen to stimulate antiserum production. This requires picking the best sequence out of an ORF of hundreds of amino acids and preparing the conjugate for injection into an animal for generation of the antiserum (*UNIT 18.3*).

Units planned for future expansion of this chapter will cover synthesis of derivatives such as phosphopeptides or other modification that can be done at the resin-bound stage, the

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reactions necessary to form disulfide bridges at the appropriate points in peptides, the chemistry that has been developed to permit the ligation of two relatively large peptide units together, the creation of multiple antigenic peptides (branched peptides containing many antigens on one synthetic carrier), the creation of derivatives in which selective peptide bonds have been replaced to create inhibitors or to stabilize against degradation in biological systems, methods for storage and handling of peptides, specialized methods for different types of resins for solid-phase synthesis, the creation of peptide libraries by chemical or biological approaches, the chemistry necessary to achieve the coupling of difficult or hindered peptides, and methods for the assembly of peptides of 100 amino acid residues in a single, long synthesis.

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Introduction to Peptide Synthesis

DEVELOPMENT OF SOLID-PHASE PEPTIDE-SYNTHESIS METHODOLOGY

A number of synthetic peptides are significant commercial or pharmaceutical products, ranging from the dipeptide sugar substitute aspartame to clinically used hormones such as oxytocin, adrenocorticotropic hormone, and calcitonin. Rapid, efficient, and reliable methodology for the chemical synthesis of these molecules is of utmost interest. The stepwise assembly of peptides from amino acid precursors has been described for nearly a century. The concept is a straightforward one, whereby peptide elongation proceeds via a coupling reaction between amino acids, followed by removal of a reversible protecting group. The first peptide synthesis, as well as the creation of the term "peptide," was reported by Fischer and Fourneau (1901). Bergmann and Zervas (1932) created the first reversible N^{α}-protecting group for peptide synthesis, the carbobenzoxy (Cbz) group. DuVigneaud successfully applied early "classical" strategies to construct a peptide with oxytocin-like activity (duVigneaud et al., 1953). Classical, or solution-phase methods for peptide synthesis have an elegant history and have been well chronicled. Solution synthesis continues to be especially valuable for large-scale manufacturing and for specialized laboratory applications.

Peptide synthesis became a more practical part of present-day scientific research following the advent of solid-phase techniques. The concept of solid-phase peptide synthesis (SPPS) is to retain chemistry that has been proven in solution but to add a covalent attachment step that links the nascent peptide chain to an insoluble polymeric support (resin). Subsequently, the anchored peptide is extended by a series of addition cycles (Fig. 18.1.1). It is the essence of the solid-phase approach that reactions are driven to completion by the use of excess soluble reagents, which can be removed by simple filtration and washing without manipulative losses. Once chain elongation has been completed, the crude peptide is released from the support.

In the early 1960s, Merrifield proposed the use of a polystyrene-based solid support for peptide synthesis. Peptides could be assembled stepwise from the C to N terminus using N^{α}-protected amino acids. SPPS of a tetrapeptide was achieved by using Cbz as an α-amino-protecting group, coupling with *N,N'*-dicyclohexylcarbodiimide (DCC), and liberating the peptide from the support by saponification or by use of HBr (Merrifield, 1963). SPPS was later modified to use the *t*-butyloxycarbonyl (Boc) group for N^{α} protection (Merrifield, 1967) and hydrogen fluoride (HF) as the reagent for removal of the peptide from the resin (Sakakibara et al., 1967). SPPS was thus based on "relative acidolysis," where the N^{α}-protecting group (Boc) was labile in the presence of moderate acid (trifluoroacetic acid; TFA), while side-chain-protecting benzyl (Bzl)–based groups and the peptide/resin linkage were stable in the presence of moderate acid and labile in the presence of strong acid (HF). The first instrument for automated synthesis of peptides, based on Boc SPPS, was built by Merrifield, Stewart, and Jernberg (Merrifield et al., 1966), and is presently on display at the Smithsonian Institute. From the 1960s through the 1980s, Boc-based SPPS was fine-tuned (Merrifield, 1986). This strategy has been utilized for synthesis of proteins such as interleukin-3 and active enzymes including ribonuclease A and all-L and all-D forms of HIV-1 aspartyl protease.

In 1972, Carpino introduced the 9-fluorenylmethoxycarbonyl (Fmoc) group for N^{α} protection (Carpino and Han, 1972). The Fmoc group requires moderate base for removal, and thus offered a chemically mild alternative to the acid-labile Boc group. In the late 1970s, the Fmoc group was adopted for solid-phase applications. Fmoc-based strategies utilized *t*-butyl (*t*Bu)–based side-chain protection and hydroxymethylphenoxy-based linkers for peptide attachment to the resin. This was thus an "orthogonal" scheme requiring base for removal of the N^{α}-protecting group and acid for removal of the side-chain protecting groups and liberation of the peptide from the resin. The milder conditions of Fmoc chemistry as compared to Boc chemistry—which include elimination of repetitive moderate acidolysis steps and the final strong acidolysis step—were envisioned as being more compatible with the synthesis of peptides that are susceptible to acid-catalyzed side reactions. In particular, the modification of the indole ring of Trp was viewed as a particular problem during Boc-based peptide synthesis (Barany and Merrifield, 1979), which could be

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alleviated using Fmoc chemistry. One example of the potential advantage of Fmoc chemistry for the synthesis of multiple-Trp-containing peptides was in the synthesis of gramicidin A. Gramicidin A, a pentadecapeptide containing four Trp residues, had been synthesized previously in low yields (5% to 24%) using Boc chemistry. The mild conditions of Fmoc chemistry dramatically improved the yields of gramicidin A, in some cases up to 87% (Fields et al., 1989, 1990). A second multiple-Trp-containing peptide, indolicidin, was successfully assembled in high yield by Fmoc chemistry (King et al., 1990). Thus, the mild conditions of Fmoc chemistry appeared to be advantageous for certain peptides, as compared with Boc chemistry.

One of the subsequent challenges for practitioners of Fmoc chemistry was to refine the technique to allow for construction of proteins, in similar fashion to that which had been achieved with Boc chemistry. Fmoc chemistry

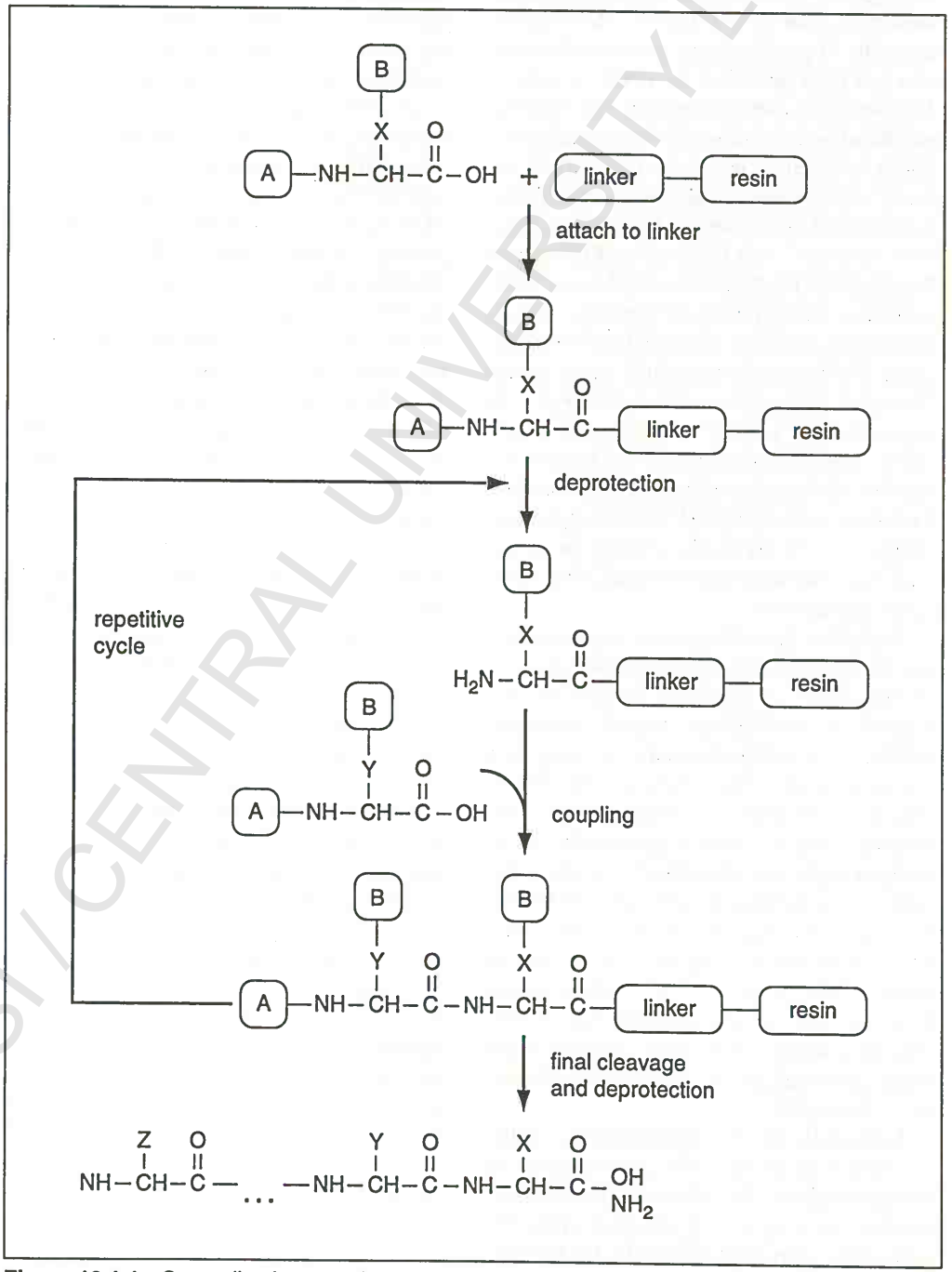

Figure 18.1.1 Generalized approach to solid-phase peptide synthesis. Symbols: A, N^{α}-protecting group; B, side-chain protecting groups; X, Y, and Z, side-chain functionalities.

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had its own set of unique problems, including suboptimum solvation of the peptide/resin, slow coupling kinetics, and base-catalyzed side reactions. Improvements in these areas of Fmoc chemistry (Atherton and Sheppard, 1987; Fields and Noble, 1990; Fields et al., 1992) allowed for the synthesis of proteins such as bovine pancreatic trypsin inhibitor analogs, ubiquitin, yeast actin-binding protein 539-588, human β-chorionic gonadotropin 1-74, and active HIV-1 protease.

The milder conditions of Fmoc chemistry, along with improvements in the basic chemistry, have led to a shift in the chemistry employed by peptide laboratories. This trend is best exemplified by a series of studies (Angeletti et al., 1997) carried out by the Peptide Synthesis Research Committee (PSRC) of the Association of Biomolecular Resource Facilities (ABRF). The PSRC was formed to evaluate the quality of the synthetic methods utilized in its member laboratories for peptide synthesis. The PSRC designed a series of studies from 1991 to 1996 to examine synthetic methods and analytical techniques. A strong shift in the chemistry utilized in core facilities was observed during this time period—i.e., the more senior Boc methodology was replaced by Fmoc chemistry. For example, in 1991 50% of the participating laboratories used Fmoc chemistry, while 50% used Boc-based methods. By 1994, 98% of participating laboratories were using Fmoc chemistry. This percentage remained constant in 1995 and 1996. In addition, the overall quality of the peptides synthesized improved greatly from 1991 to 1994. Possible reasons for the improved results were any combination of the following (Angeletti et al., 1997):

1. The greater percentage of peptides synthesized by Fmoc chemistry, where cleavage conditions are less harsh;
2. The use of different side-chain protecting group strategies that help reduce side reactions during cleavage;
3. The use of cleavage protocols designed to minimize side reactions;
4. More rigor and care in laboratory techniques.

The present level of refinement of solid-phase methodology has led to numerous, commercially available instruments for peptide synthesis (Table 18.1.1).

The next step in the development of solid-phase techniques includes applications for peptides containing non-native amino acids, post-translationally modified amino acids, and pseudoamino acids, as well as for organic molecules in general. Several areas of solid-phase synthesis need to be refined to allow for the successful construction of this next generation of biomolecules. The solid support must be versatile so that a great variety of solvents can be used, particularly for organic-molecule applications. Coupling reagents must be sufficiently rapid so that sterically hindered amino acids can be incorporated. Construction of peptides that contain amino acids bearing post-translational modifications should take advantage of the solid-phase approach. Finally, appropriate analytical techniques are needed to assure the proper composition of products.

THE SOLID SUPPORT

Effective solvation of the peptide/resin is perhaps the most crucial condition for efficient chain assembly during solid-phase synthesis. Swollen resin beads may be reacted and washed batch-wise with agitation, then filtered either with suction or under positive nitrogen pressure. Alternatively, they may be packed in columns and utilized in a continuous-flow mode by pumping reagents and solvents through the resin. ^{1}H, ^{2}H, ^{13}C, and ^{19}F nuclear magnetic resonance (NMR) experiments have shown that, under proper solvation conditions, the linear polystyrene chains of copoly(styrene-1%-divinylbenzene) resin (PS) are nearly as accessible to reagents as if free in solution. ^{13}C and ^{19}F NMR studies of Pepsyn (copolymerized dimethylacrylamide, *N,N'*-bisacryloylethylenediamine, and acryloylsarcosine methyl ester) have shown similar mobilities at resin-reactive sites as PS. Additional supports created by grafting polyethylene glycol (polyoxyethylene) onto PS—either by controlled anionic polymerization of ethylene oxide on tetraethylene glycol–PS (POE-PS) or by coupling N^{ω}-Boc– or Fmoc–polyethylene glycol acid or –polyethylene glycol diacid to amino-functionalized PS (PEG-PS)—combine the advantages of liquid-phase synthesis (i.e., a homogeneous reaction environment) and solid-phase synthesis (an insoluble support). ^{13}C NMR measurements of POE-PS showed the polyoxyethylene chains to be more mobile than the PS matrix, with the highest T_1 spin-lattice relaxation times observed with POE of molecular weight 2000 to 3000. Other supports that have been developed that show improved solvation properties and/or are applicable to organic synthesis include polyethylene glycol polyacrylamide (PEGA), cross-linked acrylate ethoxylate resin (CLEAR), and augmented surface polyethylene prepared by chemical trans-

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Table 18.1.1 Instruments for Solid-Phase Synthesis

Supplier[a]	Instrument model	Fmoc	Boc	Batch	Flow	Monitoring	Scale	No. of peptides
Peptide synthesis systems								
Advanced ChemTech	90	Yes	Yes	Yes	No	No	0.1-12 mmol	1 or 2
	396MBS	Yes	Yes	Yes	No	No	0.005-1 mmol	96
	400	Yes	Yes	Yes	No	No	25 mmol	1
	348	Yes	Yes	Yes	No	No	5-150 µmol	48
	357	Yes	Yes	Yes	No	No	5-250 µmol	42
Bachem Bioscience	SP4000-LAB	Yes	Yes	Yes	No	No	0.25-5 mmol	1
	SP4000-PRO	Yes	No	Yes	No	No	5-50 mmol	1
Gilson/Abimed	AMS422	Yes	No	Yes	No	Yes	0.005-1 mmol	1
Perkin-Elmer	ABI432A	Yes	No	No	Yes	Yes	25 µmol	1
	ABI433A	Yes	No	Yes	No	Yes	0.05-1 mmol	1
PerSeptive Biosystems	Pioneer-MPS	Yes	No	No	Yes	Yes	5-100 µmol	16
	9050 Plus	Yes	No	No	Yes	Yes	0.05-1 mmol	1
Rainin	PS3	Yes	Yes	Yes	No	No	0.1-0.25 mmol	3
	Sonata/Pilot	Yes	Yes	Yes	No	No	0.1-50 mmol	1
	Symphony/Multiples	Yes	No	Yes	No	No	5-350 µmol	12
CS Bio	CS100	Yes	Yes	Yes	No	No	0.05-1 mmol	1
	System 10	Yes	Yes	Yes	No	No	0.1-0.5 mmol	10
	036	Yes	Yes	Yes	No	No	0.1-2.5 mmol	1
	136	Yes	Yes	Yes	No	No	0.1-2.5 mmol	1
Tyler Research	SAMPS	Yes	No	Yes	No	No	10-200 µmol	100
Multiple organic synthesis units								
CS Bio	CS536	Yes	Yes	Yes	No	No	0.2-25 mmol	1
	CS936S	Yes	Yes	Yes	No	No	Up to 600 mmol	1
	CS936	Yes	Yes	Yes	No	No	Up to 12.5 mol	1
Advanced ChemTech	384	Yes	Yes	Yes	No	No	—	4 × 96
	440	Yes	Yes	Yes	No	No	—	40
	496	Yes	Yes	Yes	No	No	—	96

[a]For contact information, see *SUPPLIERS APPENDIX*.

formation (ASPECT). As the solid-phase method has expanded to include organic-molecule and library syntheses, the diversity of supports will enhance the efficiency of these new applications.

Successful syntheses of problematic sequences can be achieved by manipulation of the solid support. In general, the longer the synthesis, the more polar the peptide/resin will become (Sarin et al., 1980). One can alter the solvent environment and enhance coupling efficiencies by adding polar solvents and/or chaotropic agents (Fields and Fields, 1994). Also, using a lower substitution level of resin to avoid interchain crowding can improve the synthesis (Tam and Lu, 1995). During difficult syntheses, deprotection of the Fmoc group can proceed slowly. By spectrophotometrically monitoring deprotection as the synthesis proceeds, one can detect problems and extend base-deprotection times and/or alter solvation conditions as necessary.

COUPLING REAGENTS

The classical examples of in situ coupling reagents are *N,N'*-dicyclohexylcarbodiimide (DCC) and the related *N,N*-diisopropylcarbodiimide (Rich and Singh, 1979). The generality of carbodiimide-mediated couplings is extended significantly by the use of 1-hydroxybenzotriazole (HOBt) as an additive, which accelerates carbodiimide-mediated couplings, suppresses racemization, and inhibits dehydration of the carboxamide side chains of Asn and Gln to the corresponding nitriles. Protocols involving the in situ reagents benzotriazol-1-y'-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), *O*-benzotriazol-

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Table 18.1.2 Coupling Reagents and Additives Used in Solid-Phase Peptide Synthesis and Suppliers

Reagent	Abbreviation	Supplier(s)[a]
N,N-dicyclohexylcarbodiimide	DCC	A, ACT, AO, CI, F, PI, PL, RB, S
N,N-diisopropylcarbodiimide	DIPCDI	A, ACT, AO, CI, F, PB, RB, S
O-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate	HBTU	A, ACT, AS, CI, F, NS, PI, PL, RB, S
O-benzotriazol-1-yl-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate	TBTU	A, ACT, B, CI, CN, F, NS, PB, PI, PL, RB, S
O-(7-aza-benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate	HATU	PB
Benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate	BOP	A, ACT, AO, B, CI, CN, F, NS, PL, PI, RB, S
Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate	PyBOP	A, ACT, AO, CI, CN, F, S
7-azabenzotriazole-1-yl oxytris(pyrodidino)-phophonium hexafluorophosphate	PyAOP	PB
Tetramethylfluoroformamidinium hexafluorophosphate	TFFH	PB
1-hydroxybenzotriazole	HOBt	A, ACT, AO, AS, B, CI, NS, PB, PI, RB, S
1-hydroxy-7-azabenzotriazole	HOAt	PB
N,N-diisopropylethylamine	DIEA	A, ACT, AO, CI, F, PE, RB
N-methylmorpholine	NMM	A, AO, CI, F, S

[a]Abbreviations: A, Aldrich; ACT, Advanced ChemTech; AO, Acros Organics; AS, AnaSpec; B, Bachem; CI, Chem-Impex; CN, Calbiochem-Novabiochem; F, Fluka; NS, Neosystem/SNPE; PB, PerSeptive Biosystems; PE, Perkin-Elmer; PI, Peptides International; PL, Peninsula Laboratories; RB; Richelieu Biotechnologies; S, Sigma. For contact information, see *SUPPLIERS APPENDIX*.

1-yl-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU), and *O*-(7-aza-benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) result in coupling kinetics even more rapid than that obtained with carbodiimides. Amino acid halides have also been applied to solid-phase peptide synthesis (SPPS). N^{α}-protected amino acid chlorides have a long history of use in solution synthesis. Fmoc–amino acid chlorides and fluorides react rapidly under SPPS conditions in the presence of HOBt/*N,N*-diisopropylethylamine (DIEA) and DIEA, respectively, with very low levels of racemization. For convenience, tetramethylfluoroformamidinium hexafluorophosphate (TFFH) can be used for automated preparation of Fmoc–amino acid fluorides. Amino acid fluorides have been found to be especially useful for the preparation of peptides containing sterically hindered amino acids, such as peptaibols. All of the coupling reagents and additives discussed here are commercially available (see Table 18.1.2).

SYNTHESIS OF MODIFIED RESIDUES AND STRUCTURES

Peptides of biological interest often include structural elements beyond the 20 genetically encoded amino acids. Particular emphasis has been placed on peptides containing phosphorylated or glycosylated residues or disulfide bridges. Incorporation of side-chain-phosphorylated Ser and Thr by solid-phase peptide synthesis (SPPS) is especially challenging, as the phosphate group is decomposed by strong acid and lost with base in a β-elimination process. Boc-Ser(PO_3phenyl$_2$) and Boc-Thr(PO_3phenyl$_2$) have been found to be useful derivatives, where hydrogen fluoride (HF) or hydrogenolysis cleaves the peptide/resin and hydrogenolysis removes the phenyl groups. Fmoc-Ser(PO_3Bzl,H) can be used in conjunction with Fmoc chemistry with some care. Alternatively, peptide/resins that were built up by Fmoc chemistry to include unprotected Ser or Thr side chains may be subject to "global" or post-assembly phosphorylation. Side-chain-phosphorylated Tyr is less susceptible to strong-acid decomposition and is not at all base-labile. Thus, SPPS has been used to incor-

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porate directly Boc-Tyr(PO_3methyl$_2$), Fmoc-Tyr(PO_3tBu$_2$), and Fmoc-Tyr(PO_3H_2).

Methodology for site-specific incorporation of carbohydrates during chemical synthesis of peptides has developed rapidly. The mild conditions of Fmoc chemistry are more suited for glycopeptide syntheses than Boc chemistry, as repetitive acid treatments can be detrimental to sugar linkages. Fmoc-Ser, -Thr, -Hyp (-hydroxyproline), and -Asn have all been incorporated successfully with glycosylated side chains. The side-chain glycosyl is usually hydroxyl-protected by either benzyl (Bzl) or acetyl groups, although some SPPSs have been successful with no protection of glycosyl hydroxyl groups. Deacetylation and debenzylation are performed with hydrazine/methanol prior to glycopeptide/resin cleavage.

Disulfide-bond formation has been achieved on the solid-phase by air, $K_3Fe(CN)_6$, dithiobis(2-nitrobenzoic acid), or diiodoethane oxidation of free sulfhydryls, by direct deprotection/oxidation of Cys(acetamidomethyl) residues using thallium trifluoroacetate or I_2, by direct conversion of Cys(9-fluorenylmethyl) residues using piperidine, and by nucleophilic attack by a free sulfhydryl on either Cys(3-nitro-2-pyridinesulfenyl) or Cys(*S*-carboxymethylsulfenyl). The most generally applicable and efficient of these methods is direct conversion of Cys(acetamidomethyl) residues by thallium trifluoroacetate.

Peptides may be synthesized on a scaffold that, following cleavage and deprotection, is used directly for the production of anti-peptide antibodies. This scaffold, consisting of branched Lys residues, is referred to as a multiple antigen peptide system (MAP; Tam, 1988). MAPs may be prepared by either Boc or Fmoc chemistries.

ANALYSIS OF SYNTHETIC PEPTIDES

Each synthetic procedure has limitations, and even in the hands of highly experienced workers certain sequences defy facile preparation. The maturation of high-performance liquid chromatography (HPLC) has been a major boon to modern peptide synthesis because the resolving power of this technique facilitates removal of many of the systematic low-level by-products that accrue during chain assembly and upon cleavage. The homogeneity of synthetic materials should be checked by at least two chromatographic or electrophoretic techniques—e.g., reversed-phase HPLC (RP-HPLC; *UNIT 11.6*), ion-exchange HPLC (*UNIT 8.2*), and capillary zone electrophoresis (*UNIT 10.9*). Also, determination of a molecular ion by mass spectrometry (MS; see Chapter 16) using a mild ionization method is important for proof of structure. Synthetic peptides must be checked for the proper amino acid composition, and in some cases sequencing data are helpful. The Peptide Synthesis Research Committee (PSRC) studies (see discussion of Development of Solid-Phase Peptide Synthesis Methodology) allowed for a side-by-side comparison of a variety of analytical techniques. Efficient characterization of synthetic peptides was best obtained by a combination of RP-HPLC and MS, with sequencing by either Edman degradation sequence analysis or tandem MS (*UNIT 16.1*) being used to identify the positions of modifications and deletions. Proper peptide characterization by multiple techniques is essential.

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Contributed by Gregg B. Fields
University of Minnesota
Minneapolis, Minnesota

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Synthesis of Multiple Peptides on Plastic Pins

Scanning protein sequences by bioassay for smaller bioactive peptide sequences requires a source of many peptides homologous with the parent protein sequence. This unit deals with one of the synthetic methods for making such sets of peptides (see Fig. 18.2.1). The key to preparing large numbers (hundreds to thousands) of synthetic peptides in a short time and at minimal cost is to use a parallel synthesis technique which is efficient and can be done on a small scale. The multipin technology is suitable because it can be performed without expensive synthesizers and it uses equipment available to most laboratories. Prior experience with organic synthesis techniques or peptide chemistry is useful but not essential. The products of synthesis by multipin technology are unpurified peptides which are useful as screening reagents and may also be used to prepare purified peptide on a small scale.

Most multipin techniques exploit the conventional 8 × 12 matrix layout of common microtiter equipment which simplifies handling of the synthesis, the products (peptides), and the test results. Computer assistance with synthesis and data analysis also speeds the cycle from designing the experiment through analyzing the results.

With multipin technology, peptides are synthesized in parallel on plastic "pins" (Fig. 18.2.2) to give sets of peptides suitable not only for B and T cell epitope scanning but also for other bioassays. Peptides can be either permanently bound to the surface of the plastic for direct binding assays, or they can be released into solution. There is a choice of N- and C-terminal peptide endings. For solution-phase peptides, the synthesis scale can be 1 or 5 μmol (for a 10-mer, ~1 mg or 5 mg, respectively). The preferred coupling/deprotection chemistry used is the milder 9-fluorenylmethyloxycarbonyl (Fmoc) protection scheme rather than the older *t*-butyloxycarbonyl (*t*-Boc) protection scheme (see *UNIT 18.1*), thus reducing the level of chemical safety risk arising from synthetic peptide chemistry.

This unit covers the strategy of the multiple peptide approach to biological scanning, the synthetic protocols, and the handling of peptides after synthesis—cleavage, preliminary purification, storage, and analysis (see Basic Protocol). It is specific for the multipin technique using equipment obtained from Chiron Technologies, although some of the approaches are applicable to other multiple synthesis techniques. Procedures for multipin equipment obtained from other suppliers may differ from the procedures described here, and the manufacturer's literature should be consulted. This unit also includes protocols for preparing Fmoc–amino acid solutions (see Support Protocol 1) and for acetylating (see Support Protocol 2) or biotinylating (see Support Protocol 3) synthesized peptides.

STRATEGIC PLANNING

For a protein whose primary structure is known, the conceptually simplest method of locating all the bioactive linear peptide sequences is to make all possible peptide subsets of the protein sequence and test them. If only selected parts of the sequence are synthesized, or only the predicted active parts, bioactive sequences could be missed. The use of a set of highly overlapping peptides likewise reduces the possibility that the most bioactive sequences might be missed because they are absent from the set. A set of all overlapping 20-mers offset along the sequence by one residue at a time should capture the entire set of, for example, helper T cell epitopes, and this is a much more reliable approach than trying to predict motifs. In reality, a synthetic peptide scan through a protein is a compromise between the cost and effort in making and screening all peptides and the

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need for completeness. Thus, one worker may choose to make all overlapping 8-mers to find the linear (continuous) B cell epitopes, and another may make 12-mers offset along the sequence by five residues for the same purpose. In each case, all sequences of eight residues from the protein are present in at least one peptide, but the latter approach requires only one-fifth the number of peptides.

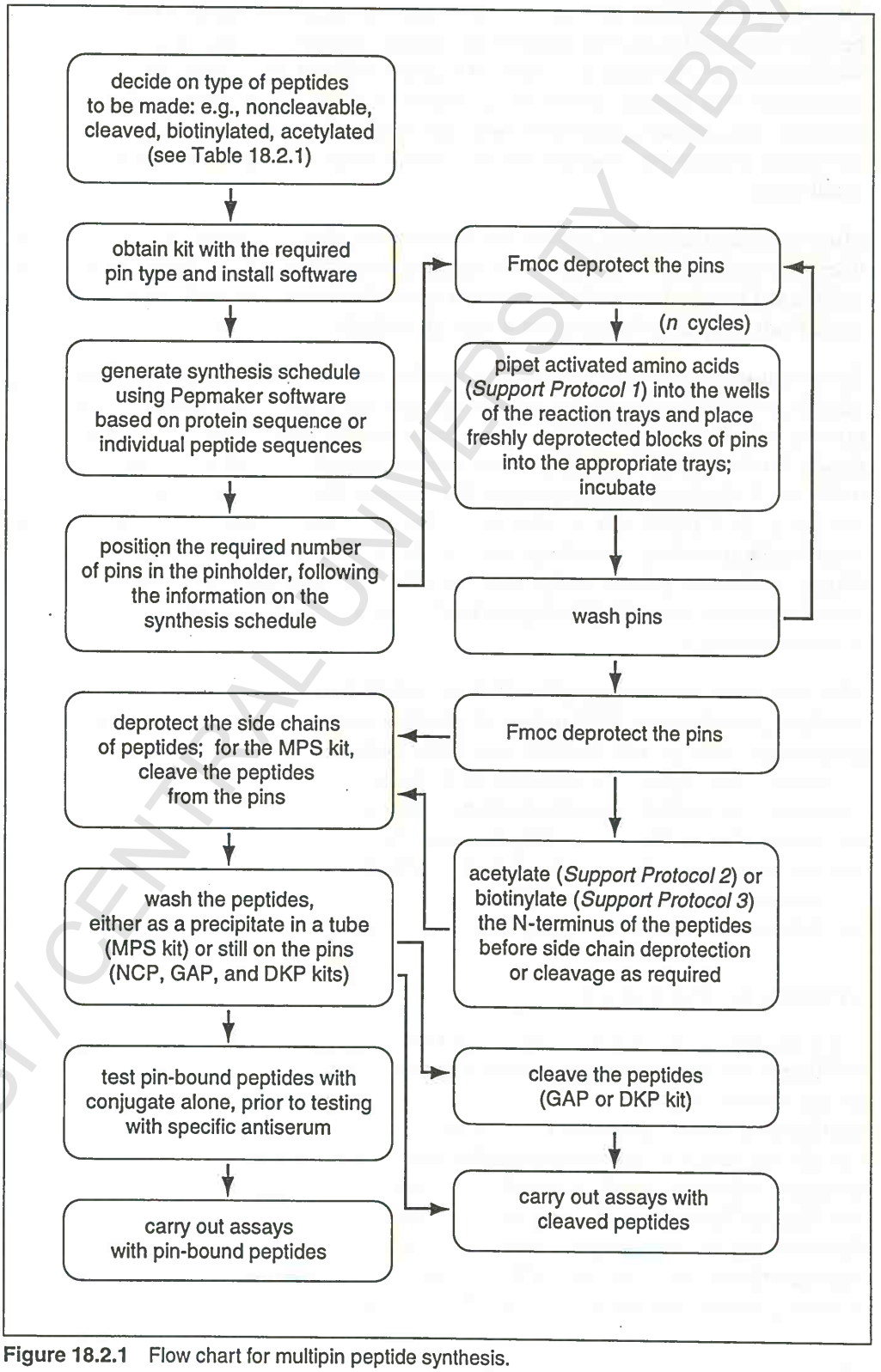

Figure 18.2.1 Flow chart for multipin peptide synthesis.

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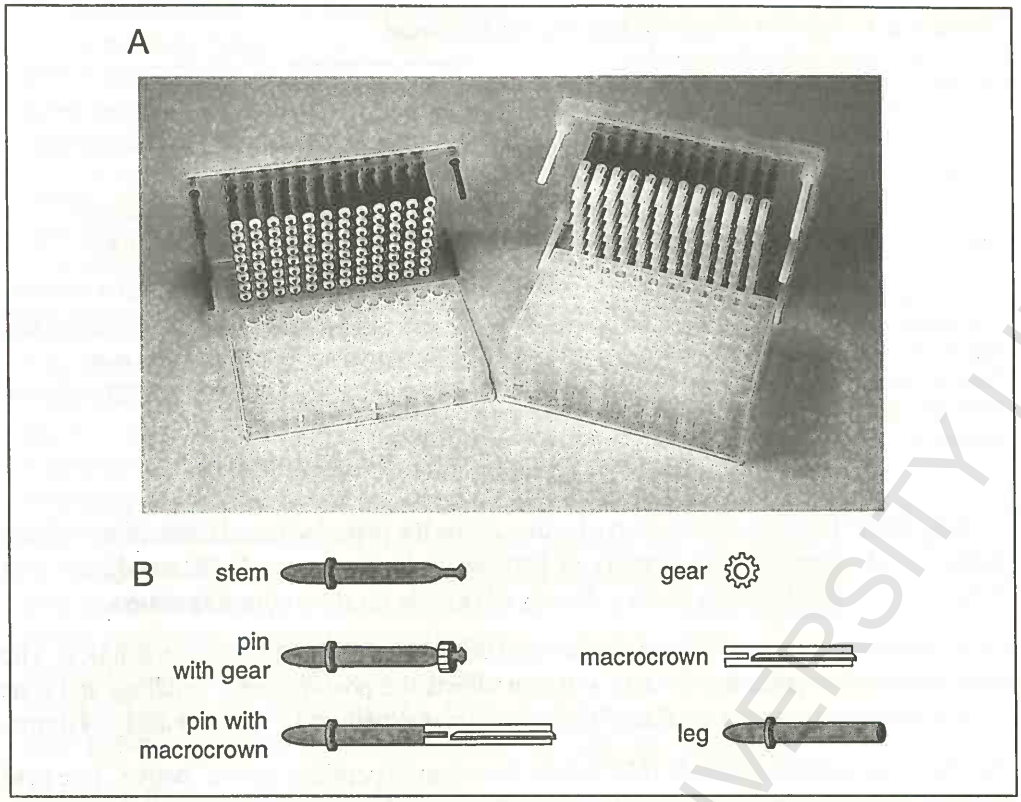

Figure 18.2.2 Apparatus for multipin peptide synthesis. (**A**) Assembled synthesis block with 96 gears (left) or 96 macrocrowns (right). (**B**) Components of the pin assembly. Components are either push-fitted together (e.g., legs or stems into the pin holder) or clipped on (gears or macrocrowns onto stems). All components are solvent-resistant plastic, either polyethylene, polypropylene, or copolymers of these two monomer types.

Planning the Synthesis

Synthetic peptides are assembled by solid-phase synthesis one amino acid at a time, commencing with the C-terminal end of the peptide on the solid phase (see *UNIT 18.1*).

The assembly process, or coupling, requires activation of the α-carboxyl group of each incoming amino acid to make it reactive with the α-amino group of the growing peptide chain. To prevent unwanted polymerization or side reaction, reactive groups in each amino acid must be temporarily protected, and the protecting group removed before further reaction can be carried out. The protecting group on the α-amino function of the most recently added amino acid must be removed before another amino acid can be coupled to it, so the α-amino protection must be labile under conditions that do not remove side-chain protection. Later, the side-chain-protecting groups must be removable under conditions that do not attack the peptide bonds. The two common protecting group "schemes" are known as *t*-butoxycarbonyl (*t*-Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc). The protecting group scheme currently recommended for multipin peptide synthesis is the milder Fmoc scheme, which is the only scheme described in this chapter.

Before beginning to plan the actual synthesis in detail, a choice needs to be made regarding how the peptides will eventually be presented in the bioassay. The options available to investigators are listed in Table 18.2.1.

For noncleavable peptide (NCP) kits, peptides are permanently bound on the solid phase (pin surface) and can be used for direct binding assays but not for interaction with living cells or other complex (e.g., multicomponent) systems. In this case, the peptides must be

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Table 18.2.1 Types of Pins for Multipin Peptide Synthesis[a]

Name	Linker[b]	Physical format[c]	Loading	Final form of peptide
NCP	Noncleavable	Gear	50 nmol	(N-capping)-PEPTIDE-linker-pin
MPS	AA ester	Macrocrown	5 μmol	(N-capping)-PEPTIDE-acid
MPS	Rink amide	Macrocrown	5 μmol	(N-capping)-PEPTIDE-amide
DKP	DKP-forming	Gear	1 μmol	(N-capping)-PEPTIDE-DKP
GAP	Glycine ester	Gear	1 μmol	(N-capping)-PEPTIDE-glycine-acid

[a]Abbreviations: DKP, diketopiperazine; GAP, glycine acid peptide; MPS, multiple peptide synthesis; NCP, noncleavable peptide; (N-capping), a free amine, acetyl group, or biotin; PEPTIDE, the sequence of the peptide being made.

[b]Nature of linker between peptide and graft polymer on the pin: noncleavable linker, β-alanine-hexamethylenediamine; DKP, diketopiperazine; AA ester, amino acid ester; Rink amide, Rink amide–forming linker.

[c]See Figure 18.2.2B.

"regenerated" between repeat assays by disrupting the peptide-ligand interaction without damaging the peptide. The quantity of peptide made is very small (50 nmol), but it is sufficient to provide a high surface density of peptide for direct binding assays.

In the other options, peptides are synthesized on pins and then released into solution. The mechanism of peptide release into solution affects the postsynthesis handling and thus the suitability of peptides produced by each cleavage method for various assay systems.

For multiple peptide synthesis (MPS) kits, the released peptides have a "native" free acid or an amide carboxy terminus. To make free acid C-termini, it is necessary to use macrocrowns that already have the first (C-terminal) amino acid on them because the chemistry of forming the first (ester) link is too difficult for the inexperienced user. In contrast, the Rink amide linker allows formation of a peptide with a C-terminal amide of any amino acid by adding the C-terminal amino acid to the Rink handle macrocrown using the standard amino acid coupling protocol. A Rink amide linker is a linker that can accept an amino acid but then can be cleaved in trifluoroacetic acid (TFA) to release the amide form of that amino acid (Rink, 1987). Although acid or amine endings are often the most desirable peptide format to have, they are also the most complex to produce because the cleavage of the peptides from the pin is into neat TFA plus scavengers which needs to be evaporated to recover the peptide. The scale of peptide synthesis for MPS kits is 5 μmol (~5 mg of a decamer).

For glycine acid peptide (GAP) kits, peptides with a glycine at the carboxy terminus are cleaved as the free acid, so that the C-terminal residue is a natural amino acid (glycine) and is not blocked. The peptides are also relatively simple to release from the pin and require little postsynthesis handling. However, the presence of glycine at the C-terminus may be undesirable where the C-terminus plays an important role in peptide bioactivity. The scale of synthesis for GAP kits is 1 μmol (~1 mg of a decamer).

In diketopiperazine (DKP) kits, peptides are synthesized with a DKP group at the C terminus. The DKP group is a cyclic dipeptide formed from C-terminal lysine and proline residues during the facile cleavage of the peptide under the mildest possible conditions: neutral aqueous buffer. In applications where the presence of the DKP group is acceptable, this type of peptide can make the downstream processing of synthetic peptides very simple and fast. The peptides can be placed into a bioassay system immediately after completing the cleavage. The scale of synthesis for DKP kits is 1 μmol (~1 mg of a decamer).

For these five kit options, it is also possible to choose a variety of N-terminal endings on the peptides. For example, it may be desirable to acetylate pin-bound peptides (see Support Protocol 2) to eliminate the positive charge that would otherwise be present on

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the α-amino group of the N-terminal residue, or to enhance the activity of a peptide in a T helper assay (Mutch et al., 1991). A handy option for cleaved peptides is to place a biotin group on the N-terminus (see Support Protocol 3) so the peptide can be captured using avidin or streptavidin. These additions must be made prior to side-chain deprotection of the peptides.

There are other configurations for multiple peptide synthesis—e.g., the SPOTS or "peptides on paper" system (Zeneca/CRB), the RaMPS system (DuPont), and multi-synthesizer machines (e.g., Advanced ChemTech).

Assessing Peptide Sequences

Peptides differ so much in properties that it is important to assess the likely properties of the peptides before attempting to synthesize them. Peptide length and hydrophobicity are the two main attributes affecting successful synthesis. The longer the peptide, the lower will be the purity of the product, as each amino acid coupling cycle is never 100% efficient. Synthesis of peptides longer than 20 residues should be avoided unless special attention can be given to each sequence. Hydrophobic peptides may be difficult to synthesize, but more significantly they may be poorly soluble in aqueous buffers, restricting their ultimate usefulness in bioassays. Prior to beginning synthesis of a set of peptides, it is sensible to assess them all for hydrophobicity (Fauchere and Pliska, 1983; *UNIT* 2.2) and decide if all should be attempted as they stand. In many cases, it is possible to choose slightly different peptides (longer, shorter, or using a different starting and finishing point in the homologous protein sequence) that will have more user-friendly properties.

As well as these general factors affecting peptides, particular peptide sequences may have characteristics that make them difficult to synthesize, or they may be problematic after synthesis. It is not feasible to discuss all the common problems here. To help assessment of peptide sequences, a software application called Pinsoft is available free from Chiron Technologies. This allows any sequence to be typed in, and an assessment is automatically reported.

Generating Peptide Sequences

Computer software (Pepmaker) supplied with synthesis kits allows sets of overlapping peptide sequences to be generated from a protein sequence computer file using the single-letter amino acid code. Alternatively, sequences can be created using a word processor and the resulting computer text file can then be used by Pepmaker to guide synthesis. The use of this software simplifies the otherwise complex and tedious task of adding the right amino acids to each reaction plate on each synthesis cycle.

MULTIPIN SYNTHESIS OF PEPTIDES

BASIC PROTOCOL

Derivatized pins with macrocrowns or gears are attached to a pin holder. Each peptide is built up on the reactive surface of one pin by successive cycles of amino acid coupling, followed by washing and removal of the 9-fluorenylmethyloxycarbonyl (Fmoc) amino-protecting group to prepare for the next amino acid coupling cycle. A critical step is properly dispensing activated amino acid solutions into the appropriate wells of each reaction tray. A list of the well locations for dispensing of each amino acid is generated by the Pepmaker software for this purpose. When the peptides are complete, trifluoroacetic acid (TFA) that contains scavengers is used to remove the side-chain-protecting groups, and for MPS kits, to cleave the peptides from the pins. The manual provided with each type of kit (see Table 18.2.1) includes instructions and hints for kit-specific procedures.

NOTE: All reagents should be of the highest grade possible, preferably peptide synthesis or analytical reagent grade.

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Materials

20% (v/v) piperidine/dimethylformamide (DMF; see recipe)
DMF, analytical reagent grade
Methanol, analytical reagent grade
100 mM activated 9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acid solutions (see Support Protocol 1)
Side chain deprotecting (SCD) solution (see recipe)
Acidified methanol: 0.5% (v/v) glacial acetic acid/methanol
1:2:0.003 (v/v/v) ether/petroleum ether/2-mercaptoethanol (2-ME)
1:2 (v/v) ether/petroleum ether
0.1 M NaOH
0.1 M acetic acid
0.1 M sodium phosphate buffer, pH 8.0 (*APPENDIX 2E*)
Sonication buffer (see recipe)

Peptide Synthesis Starter Kit (e.g., Chiron Technologies) of the desired type, containing:
  Pepmaker computer program and ELISA reading and plotting programs
  Manual
  Pins with gears or macrocrowns
Storage boxes or sealable bags, polyethylene or polypropylene (ICN Biomedicals)
Pipettor tips, polyethylene or polypropylene (ICN Biomedicals)
0.3- or 1.5-ml reaction trays, polyethylene or polypropylene (Chiron Technologies, Nunc, or Beckman)
Sonicator with power output of ~500 W
Dry nitrogen
Rack containing 96 1-ml polypropylene tubes (Bio-Rad)
10-ml capped conical polypropylene centrifuge tubes

Additional reagents and equipment for N-terminal acetylation (see Support Protocol 2; optional) or biotinylation (see Support Protocol 3; optional)

CAUTION: Perform all chemistry steps in a well-functioning chemical fume hood. Wear solvent-resistant gloves, safety glasses, and protective clothing. The reagents can be flammable, toxic, and/or carcinogenic. Avoid sources of contamination which may affect the pins, including direct contact with the bench surface or exposure to vapors. The reagents for multipin synthesis can be handled in unsealed systems, but the amount of time that these reagents are left exposed to the open air should be minimized by using capped containers for liquids or polyethylene bags for pins wherever practical. Local regulations for safe disposal of solvents and used reagents must be followed.

Prepare synthesis schedule and equipment

1. Use the Pepmaker computer program according to the instructions to generate the required set of peptide sequences (Fig. 18.2.3). Generate the printouts, which show for each coupling cycle how much of each amino acid solution, catalyst, and activating agent needs to be prepared (see Fig. 18.2.4) and where each amino acid solution is to be added to the reaction tray (see Fig. 18.2.5).

   The standard microtiter plate layout is an 8 × 12 matrix, in which the eight rows are identified as A through H and the twelve columns are identified as 1 through 12. However, the Pepmaker software uses a designation in which the column number is given first followed by a number designation for the row, beginning with row H, given in parentheses—i.e., 1(1) for well H1, 1(2) for well G1, 2(1) for well H2, and 12(8) for well A12 (see Fig. 18.2.6).

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2. Label each pin holder block indelibly on the top (e.g., Synthesis #1, Block A, Synthesis #1, Block B, and so forth), preferably by scratching into the plastic with a sharp tool. Place the label where it will help orient the block so that the pins are not accidentally placed into amino acid solutions in an inverted orientation. For example, keep pin H1 and well H1 at the lower left corner of the block (Fig. 18.2.6).

Ink labels will run or disappear with exposure to solvents.

The multipin system is based on the standard microtiter plate layout. The ***block*** *is the complete unit and consists of the* ***pin holder****, which is the support that holds 96* ***pins*** *(in an 8 × 12 array with standard ELISA microtiter plate spacing), and five* ***legs*** *to support the device and correctly position the active surfaces. A pin consists of an inert* ***stem*** *that supports either a* ***gear*** *or a* ***macrocrown****, both of which have an active surface on which the peptide is synthesized (see Figure 18.2.2). A gear is a detachable gear-shaped unit that fits on the thin end of a stem. A macrocrown is a detachable, vaned tip that fits on the thin end of a stem. It is made of high-density polyethylene and the surface is derivatized to give a solvent-compatible polymer matrix. Macrocrowns are provided in two forms: one has a linker that cleaves to give peptides with an amide at the carboxy terminus; the other has a linker that cleaves to give the free acid at the carboxy terminus and is supplied with an amino acid already attached to the linker. The* ***reaction tray*** *used for the synthesis is a polyethylene or polypropylene tray consisting of 96 wells in the standard microtiter plate 8 × 12 matrix. Shallow reaction trays (0.3-ml) are used with gears and deep trays (1.5-ml) are used with macrocrowns.*

3. Remove any pins that are not required for the first cycle of amino acid coupling and store them dry in a plastic bag in the refrigerator until needed.

Some pins need to be removed when the peptides in the synthesis are of various lengths because the software is designed to arrange all peptides to complete their synthesis on the same (final) coupling cycle. This approach eliminates unnecessary Fmoc–deprotection cycles for pins that are designated to carry the shorter peptides. The synthesis printout from the Pepmaker software shows which pins need to be added for each cycle of amino acid addition (Fig. 18.2.5). Pins (stems) can be pushed out from the top side of the pin holder. In the case of the MPS kit, where the first amino acid is already on the macrocrown as supplied, choose and mount the correct macrocrown for each position on the block.

Deprotect α-amino groups

4. Add 20% piperidine/DMF to a bath and place the pins in the bath so that the tips (macrocrowns or gears) are covered. Cover and let stand for 20 min at room temperature.

CAUTION: Piperidine is flammable.

The volume of reagent needed for all the bath steps depends on the shape of the bath, the critical factor being that all surfaces of the pins (i.e., the gears or macrocrowns) bearing the peptide need to be totally covered. A small bath suitable for gears is the upturned polypropylene lid of a pipettor tip box. For macrocrowns, deeper baths or deep-well polypropylene trays as supplied with the kit can be used.

5. Remove the block from the bath, shake off the excess liquid, and then wash the pins in a DMF bath for 2 min at room temperature.

Again, the DMF must fully cover the tips.

6. Shake off the excess DMF and immerse the block completely in a deep bath of methanol for 2 min so that all surfaces of the block are washed.

CAUTION: Methanol is flammable and toxic.

In a shallower bath the block can be turned over so that the pin holder part is washed as well.

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A

```
      GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1          Page 1
 Description: Example of a scan through Sperm Whale Myoglobin
        8-mer peptides based on the sequence MBN-SW
              Peptide spacing increment is 1
     Segment  1:  146 peptides starting at residue 1
          First peptide: [VLSEGEWQ]
          Last  peptide: [YKELGYQG]
          Protein sequence: MBN-SW (153 residues)
   1: VLSEGEWQLV  LHVWAKVEAD  VAGHGQDILI  RLFKSHPETL  EKFDRFKHLK
  51: TEAEMKASED  LKKHGVTVLT  ALGAILKKKG  HHEAELKPLA  QSHATKHKIP
 101: IKYLEFISEA  IIHVLHSRHP  GNFGADAQGA  MNKALELFRK  DIAAKYKELG
 151: YQG
              Amino Acid set to be used - AASET1
   aaset 1:Free acid L-Fmoc amino acids - DIC/HOBt chemistry
 Number of copies of each peptide  1
 Schedule based on a 250 microliter fill/well
   (Well concentration is 100 mM)
```

Figure 18.2.3 *(above and at right)* A portion of the synthesis schedule worksheets generated by Pepmaker software for Schedule no. 1 for synthesis of a set of all possible overlapping octamers of sperm whale myoglobin. **(A)** This page of the synthesis schedule is a summary of the features of the protein including its sequence. **(B)** This page of the synthesis schedule shows the sequences of the first 96 peptides that will be synthesized, the first two of which are the controls, PLAQGGGG and GLAQGGGG. Peptide sequences are shown in the conventional amino-to-carboxy-terminal direction (from left to right), with a "<" sign indicating the end attached to the solid phase during synthesis. Because amino acid couplings are carried out in the carboxy-to-amino direction, the first amino acids coupled are at the right-hand end of each sequence, adjacent to the "<".

7. Place the block in a second methanol bath to fully cover the tips. Wash for 2 min. Repeat this washing step again with a fresh methanol bath for a total of three methanol washes.

8. Remove the block and allow it to air dry in an acid-free fume hood for a minimum of 30 min.

   Avoid exposure to acidic fumes as this could prevent efficient coupling in the next step.

   The block can be conveniently left to dry while the amino acid solutions are being dispensed.

Dispense activated amino acid solutions

9. Dispense the required volume of each activated amino acid solution (see Support Protocol 1; 150 µl for gears or 450 µl for macrocrowns) into the appropriate wells of the reaction tray as specified by the synthesis schedule for the coupling cycle (e.g., Fig. 18.2.5).

Perform the amino acid coupling

10. Place the pins in the activated amino acid solutions in the reaction tray, ensuring that the block is correctly oriented before actually lowering the pins into the solution. Incubate ≥2 hr at 20° to 25°C in a polyethylene box with a lid or in a sealable polyethylene bag.

    Coupling begins immediately and is irreversible.

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B GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1 Page 2

Amino terminus is printed on the left

```
  1 A 1(1)PLAQGGGG<      49 A 1(5)KHLKTEAE<
  2 A 2(1)GLAQGGGG<      50 A 2(5)HLKTEAEM<
  3 A 3(1)VLSEGEWQ<      51 A 3(5)LKTEAEMK<
  4 A 4(1)LSEGEWQL<      52 A 4(5)KTEAEMKA<
  5 A 5(1)SEGEWQLV<      53 A 5(5)TEAEMKAS<
  6 A 6(1)EGEWQLVL<      54 A 6(5)EAEMKASE<
  7 A 7(1)GEWQLVLH<      55 A 7(5)AEMKASED<
  8 A 8(1)EWQLVLHV<      56 A 8(5)EMKASEDL<
  9 A 9(1)WQLVLHVW<      57 A 9(5)MKASEDLK<
 10 A10(1)QLVLHVWA<      58 A10(5)KASEDLKK<
 11 A11(1)LVLHVWAK<      59 A11(5)ASEDLKKH<
 12 A12(1)VLHVWAKV<      60 A12(5)SEDLKKHG<
 13 A 1(2)LHVWAKVE<      61 A 1(6)EDLKKHGV<
 14 A 2(2)HVWAKVEA<      62 A 2(6)DLKKHGVT<
 15 A 3(2)VWAKVEAD<      63 A 3(6)LKKHGVTV<
 16 A 4(2)WAKVEADV<      64 A 4(6)KKHGVTVL<
 17 A 5(2)AKVEADVA<      65 A 5(6)KHGVTVLT<
 18 A 6(2)KVEADVAG<      66 A 6(6)HGVTVLTA<
 19 A 7(2)VEADVAGH<      67 A 7(6)GVTVLTAL<
 20 A 8(2)EADVAGHG<      68 A 8(6)VTVLTALG<
 21 A 9(2)ADVAGHGQ<      69 A 9(6)TVLTALGA<
 22 A10(2)DVAGHGQD<      70 A10(6)VLTALGAI<
 23 A11(2)VAGHGQDI<      71 A11(6)LTALGAIL<
 24 A12(2)AGHGQDIL<      72 A12(6)TALGAILK<
 25 A 1(3)GHGQDILI<      73 A 1(7)ALGAILKK<
 26 A 2(3)HGQDILIR<      74 A 2(7)LGAILKKK<
 27 A 3(3)GQDILIRL<      75 A 3(7)GAILKKKG<
 28 A 4(3)QDILIRLF<      76 A 4(7)AILKKKGH<
 29 A 5(3)DILIRLFK<      77 A 5(7)ILKKKGHH<
 30 A 6(3)ILIRLFKS<      78 A 6(7)LKKKGHHE<
 31 A 7(3)LIRLFKSH<      79 A 7(7)KKKGHHEA<
 32 A 8(3)IRLFKSHP<      80 A 8(7)KKGHHEAE<
 33 A 9(3)RLFKSHPE<      81 A 9(7)KGHHEAEL<
 34 A10(3)LFKSHPET<      82 A10(7)GHHEAELK<
 35 A11(3)FKSHPETL<      83 A11(7)HHEAELKP<
 36 A12(3)KSHPETLE<      84 A12(7)HEAELKPL<
 37 A 1(4)SHPETLEK<      85 A 1(8)EAELKPLA<
 38 A 2(4)HPETLEKF<      86 A 2(8)AELKPLAQ<
 39 A 3(4)PETLEKFD<      87 A 3(8)ELKPLAQS<
 40 A 4(4)ETLEKFDR<      88 A 4(8)LKPLAQSH<
 41 A 5(4)TLEKFDRF<      89 A 5(8)KPLAQSHA<
 42 A 6(4)LEKFDRFK<      90 A 6(8)PLAQSHAT<
 43 A 7(4)EKFDRFKH<      91 A 7(8)LAQSHATK<
 44 A 8(4)KFDRFKHL<      92 A 8(8)AQSHATKH<
 45 A 9(4)FDRFKHLK<      93 A 9(8)QSHATKHK<
 46 A10(4)DRFKHLKT<      94 A10(8)SHATKHKI<
 47 A11(4)RFKHLKTE<      95 A11(8)HATKHKIP<
 48 A12(4)FKHLKTEA<      96 A12(8)ATKHKIPI<
```

Figure 18.2.3 *(continued)*

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Wash the pins

11. Remove the block of pins from the amino acid solutions and, if the next cycle is to start immediately, place the block in a methanol bath in which the pins are immersed to half their height (tips are fully immersed). Wash with agitation for 5 min. Flick off excess methanol and allow to air dry for 2 min.

12. Place the block in a DMF bath in which the pins are immersed to half their height (tips are fully immersed). Wash with agitation for 5 min.

    If extra pins are to be added as synthesis progresses (if the peptides being made differ in length), add the pins to the appropriate spaces (see Fig. 18.2.5).

13. Repeat steps 4 through 12 for each cycle of amino acid addition. Follow the synthesis schedule for preparing Fmoc–protected amino acid solutions and for depositing

GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1 Page 3
Bulk solutions for activator and/or additives (150 wells)
Chemistry Group 1 data for synthesis coupling 1
Activator : DIC requires 567.9 mg in 9.0 ml of DMF
Additive 1: HOBt requires 816.4 mg in 35.5 ml of DMF

WEIGHTS FOR INDIVIDUAL AMINO ACID SOLUTIONS

AA	#	Amino acid description	Batch	Weight (mg) Target	Actual	DIC (ml)	HOBt (ml)
A	17	Fmoc-L-Ala-OH.H20	.....	155.2	.....	0.94	3.77
D	6	Fmoc-L-Asp(OtBu)-OH	.....	75.1	.....	0.36	1.46
E	12	Fmoc-L-Glu(OtBu)-OH.H2O	.....	150.8	.....	0.68	2.72
F	6	Fmoc-L-Phe-OH	.....	70.7	.....	0.36	1.46
G	14	Fmoc-Gly-OH	.....	116.7	.....	0.79	2.30
H	12	Fmoc-L-His(Boc)-OH.5DCM	.....	176.8	.....	0.68	2.72
I	9	Fmoc-L-Ile-OH	.....	92.3	.....	0.52	2.09
K	19	Fmoc-L-Lys(Boc)-OH	.....	245.4	.....	1.05	4.19
L	17	Fmoc-L-Leu-OH	.....	166.5	.....	0.94	3.77
M	2	Fmoc-L-Met-OH	.....	28.8	.....	0.16	0.62
N	2	Fmoc-L-Asn(Trt)-OH	.....	46.2	.....	0.16	0.62
P	4	Fmoc-L-Pro-OH	.....	43.9	.....	0.26	1.04
Q	5	Fmoc-L-Gln(trt)-OH	.....	95.4	.....	0.31	1.25
R	4	Fmoc-L-Arg(PMC)-OH.3IPE	.....	90.4	.....	0.26	1.04
S	5	Fmoc-L-Ser(tBu)-OH	.....	59.9	.....	0.31	1.25
T	5	Fmoc-L-Thr(tBu)-OH	.....	62.1	.....	0.31	1.25
V	7	Fmoc-L-Val-OH	.....	70.8	.....	0.42	1.67
W	1	Fmoc-L-Trp(Boc)-OH	.....	27.0	.....	0.10	0.41
Y	3	Fmoc-L-Tyr(tBu)-OH	.....	47.7	.....	0.21	0.83

Figure 18.2.4 This page of the synthesis schedule is used for the preparation of activated amino acid solutions. It shows the amounts of each amino acid (represented by the single letter code, A through Y, along the left-hand margin), activator (diisopropylcarbodiimide [DIC] in dimethylformamide [DMF]), and catalyst (additive; 1-hydroxybenzotriazole [HOBt] in DMF) needed for the first amino acid coupling cycle. (In this example the amounts are calculated for a 250-µl reaction volume.) The amino acid powder is dissolved in the HOBt/DMF solution (right-hand column) before activation with DIC/DMF solution.

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GENERAL NET SYNTHESIS SCHEDULE NUMBER : 1        Page 4
        PIN POSITIONS for Synthesis coupling  1
                        NEW PIN POSITIONS
A 1(1) TO B 6(5)

                Well positions for amino acid dispensing

A  A10(1)  A 2(2)  A 5(2)  A12(4)  A 4(5)  A 6(6)  A 9(6)
     A 7(7)  A 1(8)  A 5(8)  B11(1)  B 2(3)  B 4(3)  B 7(3)
     B11(3)  B 8(4) TO B 9(4)

D  A 3(2)  A10(2)  A 3(4)  A 7(5)  B 3(3)  B 6(4)

E  A 1(2)  A 9(3)  A12(3)  A11(4)  A 1(5)  A 6(5)  A 6(7)
     A 8(7)  B 6(1)  B10(1)  B 1(4)  B 1(5)

F  A 4(3)  A 2(4)  A 5(4)  B 7(1)  B12(2)  B 3(4)

G  A 1(1) TO A 2(1) A 6(2)  A 8(2)  A12(5)  A 8(6)  A 3(7)
     B 1(1) TO B 2(1) B10(2)  B 1(3)  B 6(3)  B 3(5)  B 6(5)

H  A 7(1)  A 7(2)  A 7(3)  A 7(4)  A11(5)  A 4(7) TO A 5(7)
     A 4(8)  A 8(8)  B 2(2)  B 5(2)  B 8(2)

I  A11(2)  A 1(3)  A10(6)  A10(8)  A12(8)  B 8(1)  B12(1)
    TO B 1(2) B 7(4)

K  A11(1)  A 5(3)  A 1(4)  A 6(4)  A 9(4)  A 3(5)  A 9(5)
    TO A10(5) A12(6) TO A 2(7) A10(7)  A 7(8)  A 9(8)  B 3(1)
     B10(3)  B 5(4)  B10(4)  B12(4)

L  A 4(1)  A 6(1)  A12(2)  A 3(3)  A11(3)  A 8(4)  A 8(5)
     A 4(6)  A 7(6)  A11(6)  A 9(7)  A12(7)  B 5(1)  B 4(2)
     B12(3)  B 2(4)  B 2(5)

M  A 2(5)  B 8(3)

N  B11(2)  B 9(3)

P  A 8(3)  A11(7)  A11(8)  B 9(2)

Q  A 3(1)  A 9(2)  A 2(8)  B 5(3)  B 5(5)

R  A 2(3)  A 4(4)  B 7(2)  B 4(4)

S  A 6(3)  A 5(5)  A 3(8)  B 9(1)  B 6(2)

T  A10(3)  A10(4)  A 2(6)  A 5(6)  A 6(8)

V  A 5(1)  A 8(1)  A12(1)  A 4(2)  A 1(6)  A 3(6)  B 3(2)

W  A 9(1)

Y  B 4(1)  B11(4)  B 4(5)
```

Figure 18.2.5 This page of the synthesis schedule identifies which wells of the reaction tray receive which activated amino acid solution. Each amino acid solution is identified along the left-hand margin using the single-letter amino acid code. Individual reaction trays and pin holders (A or B in this case) are identified by letters of the alphabet, and the paired numbers—e.g., 10(1) for well 10H—identify individual wells within reaction trays according to the numbering system illustrated in Figure 18.2.6.

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aliquots of the activated amino acids to the appropriate wells of the reaction tray for each cycle.

Two coupling cycles can be carried out during a normal working day, and a third coupling can be carried out overnight, so a total of three amino acids can be added to each pin during a 24-hr period.

14. Deprotect the final Fmoc amino acid by repeating steps 4 through 8. Then proceed with step 15 or step 16.

15. *Optional:* For B cell epitope scanning or for T helper cell epitope scanning, the N-terminus of the peptide can be capped by acetylation (see Support Protocol 2). To allow later recapture onto avidin, the N-terminus of the peptide can be capped with biotin or long-chain biotin (see Support Protocol 3).

Deprotect the side chains

16. Dispense the required volume of SCD solution into a bath or tubes (1.5 ml/tube) and fully immerse the peptide-bearing portion of the pins. Cover bath or cap tubes and incubate 2.5 hr at 20° to 25°C.

CAUTION: SCD solution is a toxic, corrosive liquid.

For blocks where the peptide is to remain attached to the pin during side chain deprotection (i.e., NCP, GAP, and DKP kits), side chain deprotection is carried out in a bath of reagent. For pins where the peptide is simultaneously side chain deprotected and cleaved from the pin (MPS kits), the process is carried out in individual 10-ml capped conical polypropylene centrifuge tubes that become the containers for the recovered peptide.

17a. *For NCP, GAP, or DKP kits:* Wash the pins 3 times in acidified methanol to remove the SCD solution prior to further treatment.

17b. *For MPS kit:* In a good chemical fume hood, reduce the volume of SCD solution containing cleaved peptide to ~0.1 ml either with a gentle stream of dry nitrogen gas or in a centrifugal vacuum drier (e.g., Speedvac) equipped to handle corrosive fumes. Precipitate the peptide in the remaining solution with 8 ml of 1:2:0.003 ether/petroleum ether/2-ME. Decant and discard the supernatant, and wash the

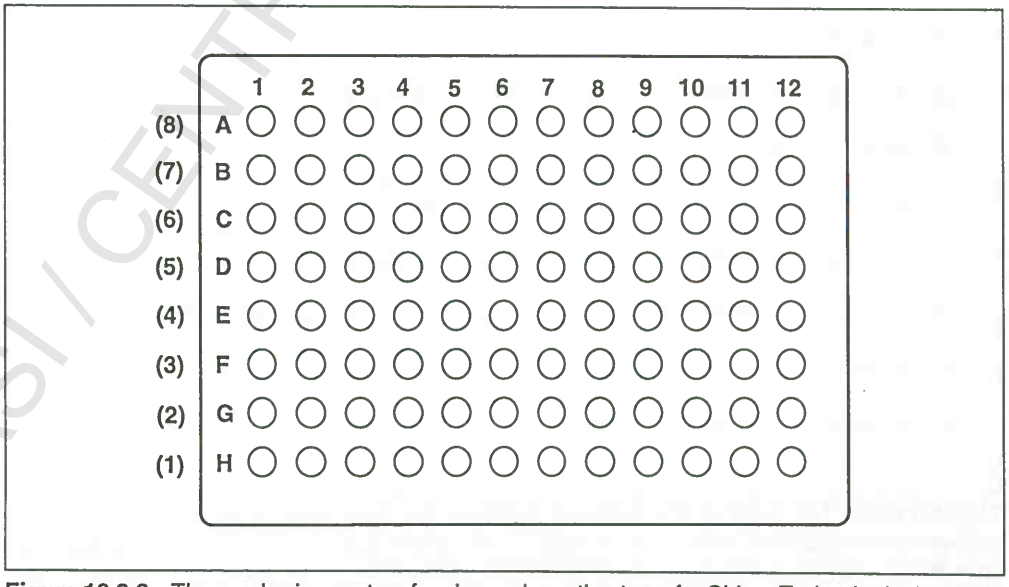

Figure 18.2.6 The numbering system for pins and reaction trays for Chiron Technologies' multipin synthesis system. Each well is identified by a pair of numbers rather than a number and a letter, e.g, 1(1). The first number identifies the column number; the second (in parentheses) identifies the lettered row, beginning with H as (1) and ending with A as (8).

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precipitated peptide with 4 ml of 1:2 ether/petroleum ether. Dry the precipitate with a gentle stream of dry nitrogen.

CAUTION: Ether/petroleum ether/2-ME and ether/petroleum ether solutions are highly flammable.

These dry peptides can now be redissolved for assay purposes or may be further purified, e.g., by HPLC (see UNIT 11.6).

Prepare the peptides

18a. *For GAP kits:* Add 0.7 ml of 0.1 M NaOH to each tube of a rack of 96 1-ml polypropylene tubes. Place the pins into the solution and incubate ~1.5 hr to cleave the peptides from the pins. Immediately after cleavage, neutralize with one equivalent of 0.1 M acetic acid.

Alternatively, the 0.1 M NaOH solution can contain 40% (v/v) acetonitrile to facilitate solubilization of the more hydrophobic peptides.

The incubation time can be shorter if the tubes are sonicated during cleavage.

18b. *For DKP kits:* Cleave the peptide from the pin in a suitable, reasonably well-buffered solution with a pH >7 overnight (16 hr).

The solution for cleavage—e.g., 0.1 M sodium phosphate, pH 8, or 0.1 M HEPES, pH 8—can be chosen to be compatible with the assay for which the peptides will be used; the solution should have a buffering capacity of ~0.05 M.

Again, an organic modifier such as acetonitrile can be added to the cleavage solution to facilitate solubilization of the more hydrophobic peptides.

18c. *For NCP kits:* Prepare the pins for binding assays by floating the block, pin-side down, in sonication buffer and sonicating 10 min at ~60°C. Rinse the pins first in water, then in 20° to 45°C methanol for immediate use in an assay or air dry the pins for storage until they are used in an assay.

PREPARING ACTIVATED Fmoc–PROTECTED AMINO ACID SOLUTIONS

SUPPORT PROTOCOL 1

Activated 9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acid solutions are prepared in two steps. First, the protected amino acid is dissolved in a solution of the catalyst, 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF). Then, just before dispensing, the amino acid is activated by adding an activating agent. The following procedure illustrates the use of diisopropylcarbodiimide (DIC) as the activating reagent. DIC is a liquid and can be measured by volume.

***Additional Materials** (also see Basic Protocol)*

9-fluorenylmethyloxycarbonyl (Fmoc)–protected amino acids with side-chain-protecting groups (Sigma, Bachem, Novachem, or Chiron Technologies), stored at 4°C
Catalyst: 1-hydroxybenzotriazole (HOBt)
Activating agent: diisopropylcarbodiimide (DIC)
Dimethylformamide (DMF), amine-free
Ethanol, analytical reagent grade
5- or 10-ml glass, polyethylene, or polypropylene bottles with inert (e.g., polythylene, Teflon) lids and liners

1. Remove the Fmoc–protected amino acids from the refrigerator and allow them to come to room temperature before weighing them out.

Warming the containers to room temperature avoids the possibility of uptake of moisture from the air onto the cold solids.

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The side chains of the amino acids must also be protected during peptide synthesis: t-butyl ether is used for serine, threonine, and tyrosine; t-butyl ester is used for aspartic acid and glutamic acid; t-butoxycarbonyl (t-Boc) is used for lysine, histidine, and tryptophan; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) is used for arginine; and trityl (Trt) is used for cysteine. If benzotriazolyl-N-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) or benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation is to be used, trityl protection should be used for asparagine and glutamine. BOP and PyBOP require greater care in handling and different protection should be used for some amino acids.

2. Weigh individual amino acids and HOBt using the quantities specified for the current synthesis cycle (e.g., see Fig. 18.2.4) into separate appropriately sized clean and dry glass, polyethylene, or polypropylene bottles. Rinse the spatula with ethanol and dry it after weighing each reagent.

   The bottle caps and inserts must be inert to any of the reagents or solvents used in making up the activated solutions (they must be Teflon, not rubber).

   Care should be taken to avoid cross-contamination of amino acids by rinsing the spatula in ethanol between weighings (the spatula must be dry before each use) and by making sure that all lids, as well as the containers, are labeled so they can be replaced on the correct bottles after weighing has been completed.

3. Measure the appropriate amount of DIC (see "Activator" as in Fig. 18.2.4).

   DIC is a liquid so it is more convenient to measure it by volume rather than by weight. Multiply the indicated weight by 1.23 (based on the density of DIC, 0.815 g/ml) as a conversion factor from the calculated weight to get the required volume of DIC in microliters.

4. Prepare HOBt and DIC solutions, by pipetting the appropriate volumes of purified (amine-free) DMF as shown on the synthesis schedule (e.g., see Fig. 18.2.4).

   Both reagents should be fully dissolved in the DMF before using them to prepare the activated amino acid solutions.

5. Add the specified volume of HOBt/DMF solution (e.g., Fig. 18.2.4, column headed "HOBt") to the individual amino acids.

   Make sure the amino acids are completely dissolved before adding the activator solution.

   Unactivated amino acid solutions may be stored a few days at 4°C.

6. Activate the individual amino acid solutions by adding the specified volume of DIC/DMF solution to each amino acid solution (e.g., Fig. 18.2.4, column headed "DIC"). Mix thoroughly and use immediately for peptide synthesis.

   Activated amino acids should be prepared immediately before use and any excess should be discarded.

SUPPORT PROTOCOL 2

N-TERMINAL ACETYLATION OF PEPTIDES

N-terminal capping of the peptides is carried out after a final 9-fluorenylmethyloxycarbonyl (Fmoc)–deprotection cycle and prior to side chain deprotection. The process is similar to coupling of amino acids, except that in the case of acetylation the active reagent can be acetic anhydride rather than acetic acid. Acetic anhydride does not require activation. If acetic anhydride is not available, simply use acetic acid as if it were an amino acid (see Support Protocol 3).

Additional Materials (also see Basic Protocol)

Acetylation solution (see recipe), prepared just before use
Pins with completed peptides (see Basic Protocol)

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1. Add freshly prepared acetylation solution to appropriate bath container.
2. Immerse the pins with completed peptides in acetylation solution and incubate 90 min at room temperature.
3. Wash the pins in a methanol bath and air dry.

The pins can now be used for side chain deprotection (Basic Protocol, step 16).

N-TERMINAL BIOTINYLATION OF PEPTIDES

SUPPORT PROTOCOL 3

Biotin can also be coupled to the N-terminus of peptides after N-terminal deprotection and before side chain deprotection. The reagent is used as if it were an amino acid, using the same solvent, activating agent, and catalyst.

***Additional Materials** (also see Basic Protocol)*

Biotin or long-chain biotin
Dimethylformamide (DMF), amine-free
Diisopropylcarbodiimide (DIC)
Pins with completed peptides (see Basic Protocol)

1. Dissolve biotin in amine-free DMF to a concentration of 125 mM.
2. Prepare a 10× solution of DIC (activating agent) by dissolving 158 mg DIC in 1 ml DMF and prepare a 10× solution of HOBt (catalyst) by dissolving 192 mg HOBt in 1 ml DMF.
3. Activate the biotin with the 10× concentrate solutions of activation agent and catalyst (80:10:10 [v/v/v]).
4. Dispense 150 µl/well (for gears) or 450 µl/well (for macrocrowns) into reaction trays.
5. Immerse the pins with completed peptides in the reaction tray and incubate ≥2 hr.
6. Wash the pins in methanol.

The pins can now be used for side chain deprotection (see Basic Protocol, step 16).

REAGENTS AND SOLUTIONS

*Use Milli-Q water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2E**; for suppliers, see **SUPPLIERS APPENDIX**.*

Acetylation solution

For 200 ml:
193 ml dimethylformamide (DMF)
6 ml acetic anhydride
1 ml *N*-ethyldiisopropylamine
Prepare immediately before use and discard after use

DO NOT expose pins to acetic anhydride at any other time except during acetylation. Also, do not store acetic anhydride anywhere near where peptide synthesis is performed.

The DMF does not need to be amine-free.

20% piperidine/DMF

Prepare a 20% (v/v) solution of the best quality piperidine available in analytical reagent–grade dimethylformamide (DMF). Prepare a fresh solution for each synthesis (solution can be reused several times within a synthesis). Store at room temperature in an amber bottle containing activated molecular sieves to remove moisture.

CAUTION: This solution is highly flammable and toxic.

continued

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If high-quality piperidine is not available, it may have to be treated with solid sodium hydroxide and redistilled.

DMF need not be amine-free.

Side chain deprotecting (SCD) solution

33 parts (v/v) trifluoroacetic acid
1 part (v/v) ethanedithiol
2 parts (v/v) anisole
2 parts (v/v) thioanisole
2 parts (v/v) H_2O
Prepare immediately before use and do not store or reuse

CAUTION: This solution is corrosive and extremely malodorous. Contamination of the laboratory, especially with ethanedithiol, should be avoided. Wipe the outside of ethanedithiol-contaminated equipment or containers with dilute, 0.1% aqueous hydrogen peroxide to oxidize ethanedithiol to a nonodorous compound before removing the container from the fume hood. DO NOT allow hydrogen peroxide to contact other readily oxidizable materials or reagents.

Sonication buffer

1% (w/v) SDS
0.1 M sodium phosphate buffer, pH 7.2 (*APPENDIX 2E*)
0.1% (v/v) 2-mercaptoethanol (2-ME)
Store at room temperature up to 1 week

CAUTION: Before discarding sonication buffer, destroy remaining 2-ME by adding 2 ml 30% hydrogen peroxide per liter of buffer.

COMMENTARY

Background Information

The multipin method was developed by Dr. H.M. Geysen and coworkers (Geysen et al., 1984, 1987) as a scanning method for linear antibody-defined epitopes. Eventually in the late 1980s, the method was adapted to parallel synthesis of cleaved (soluble) peptides (Maeji et al., 1990), opening the way for systematic scanning of T helper (Reece et al., 1993) and cytotoxic epitopes (Burrows et al., 1994). Initially only suitable for synthesis of short peptides (up to 10 amino acid residues), the method can now routinely produce peptides of up to 20 residues of acceptable quality for initial screening experiments (Valerio et al., 1993).

Critical Parameters

Successful peptide synthesis requires reagents of a quality appropriate to the particular step, and the careful application of those reagents. For example, the protected amino acids need to be free of reactive counterions such as dicyclohexylamine (DCHA), contaminating unprotected amino acid, isomers such as the D-amino acid, and water. Check carefully that the amino acid as supplied is EXACTLY the same as specified in the manual or on the software. Apart from quality testing each amino acid, the best assurance of quality is to buy only from reputable suppliers.

Dimethylformamide (DMF) is the primary solvent for carrying out reactions (couplings) on pins. Its low volatility and moderate polarity make it suitable for dissolving the amino acids and solvating the graft polymer/growing peptide on the pin surface. Purity is not critical for some (washing) steps, but is critical for the DMF used just before and during amino acid coupling. Presence of excessive amine in the DMF results in loss of activated amino acid because the amino acid couples to the amine rather than to the peptide on the pin. Fortunately, the pin system allows use of substantial molar excesses of incoming amino acid (typically 6- to 1000-fold), so loss of some amino acid is not disastrous. Fresh DMF of the best available grade should be used for the coupling, and it is recommended that the amine level be tested using the FDNB test (Stewart and Young, 1984).

Liberal use is made of methanol as a washing solvent. Analytical reagent grade methanol is readily available at low cost in large containers (20 or 200 liters) and is relatively easy to dispose of. It is possible to reduce the use of methanol by reusing it for washes: the last wash

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bath in any series should be in fresh (pure) methanol. In the next round of washes, the former last bath is then reassigned as the second-to-last wash, the previously second-to-last bath becomes the third-to-last, and so on. For each synthesis cycle, the first wash bath in the series is the one which is discarded. The presence of methanol is undesirable during reactions on the pins, but as it evaporates readily it can be easily removed by standing the block in a moving stream of air, such as the opening of an operating chemical fume hood. Methanol will dry more rapidly and the methanol-washed pins will take up less moisture from the air if the methanol is warm (e.g., prewarmed to 45°C in a closed bottle in a water bath).

Other solvents (e.g., ether, petroleum ether, acetonitrile) should be the best available grade.

Carrying out the correct synthesis of the peptides requires that all steps are performed with a very high level of attention to detail. All cyclically repeated steps (washes and deprotections) must be performed, and the activation and dispensing of the amino acids for each coupling cycle must be carried out exactly, or the peptides made may have the incorrect sequence, may be missing an amino acid, or may be truncated. Computerized equipment is available for assisting with the accurate dispensing of amino acids to the wells in a reaction tray (e.g., "Pin-Aid," Chiron Technologies; Carter et al., 1992). The growing peptides must not be subjected to conditions that would prematurely block or deprotect the side chains (for example, from premature exposure to acetic anhydride or trifluoroacetic acid, which should be stored well away from where peptide synthesis is being performed).

As a spot test for correct completion of all the steps of synthesis, it is wise to synthesize controls on each block of 96 pins. For noncleavable peptides, these controls can be peptide sequences that can be probed with an antibody known to react with the peptide. In this case, one of the two peptides should be a negative control, such as a randomized sequence. For cleavable peptides, the quantity and quality of the controls can be monitored by the usual techniques of HPLC (*UNIT 11.6*), amino acid analysis (*UNIT 11.9*), and mass spectrometry (Chapter 16). Ultimately, proof that an assay result is a function of the particular peptide made has to rely on a confirmatory experiment carried out with more highly-characterized peptide or on analysis of a sample of the particular peptide used in the experiment.

Once peptides have been made, they need to be handled and stored carefully to prevent degradation. Noncleavable peptides (pins) should be stored dry in a refrigerator after removal of any bound protein. If stored with desiccant they should be stable for months to years. Cleaved peptides can be stored frozen or as dry powder. After a long period of storage, it is wise to reassay controls or confirm the quality of the stored peptide by analysis.

Another parameter critical to data from large numbers of peptides is to ensure that the identity of each peptide is properly tracked and that activity is not ascribed to the wrong peptide. Consistent use of the 8 × 12 microtiter plate format for synthesis, storage, assay, and use of computerized records for tracking all three processes can help avoid mistakes. Tracking and control is particularly easy if the assay data is read directly from a microtiter plate reader to a computer that is programmed with the peptide information because this method avoids manual data transcription.

Anticipated Results

For a noncleavable pin-peptide synthesis, two control peptides, one of which is reactive with a monoclonal antibody in ELISA and the other serving as a nonbinding peptide control, should show the specific binding expected based on past data. For cleaved peptides, the yield of control peptide should be in the range expected from the stated pin loading (substitution level), e.g., 1 µmol for GAP and DKP kits or 5 µmol for the MPS kit. Purity of the cleaved controls should be consistent with the results of previous batches and should be of an acceptable standard.

Testing of a systematic set of peptides in a bioassay can give data that is interpretable without recourse to additional controls, because a systematic set of peptides through a protein includes many sequences that are unlikely to be reactive sequences, i.e., they act as internal negative controls. Figure 18.2.7 shows one set of ELISA data from scanning noncleaved peptides with a monoclonal antibody. In screening for T helper cell responsiveness it is critical to include many control cultures, not only controls with no peptide added but also controls with nonstimulatory peptide. Systematic sets of peptides automatically include such controls (Reece et al., 1994).

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Time Considerations

If amino acid coupling is carried out at 3 cycles/day, which can fit into a conventional working day, then it will take up to 2 weeks to make a set of 15-mers, as there is extra time required for side chain deprotection and drying down (depending on the peptide format). Although this may seem slow, the fact that hundreds or thousands of peptides can be made simultaneously means that a project requiring large numbers of peptides is completed in a very short time. Indeed, the rate-limiting step may be the time it takes to carry out the assays on the large number of peptides when they become available.

From this perspective, biotinylated peptides produced on glycine acid peptide (GAP), diketopiperazine (DKP), or multiple peptide syn-

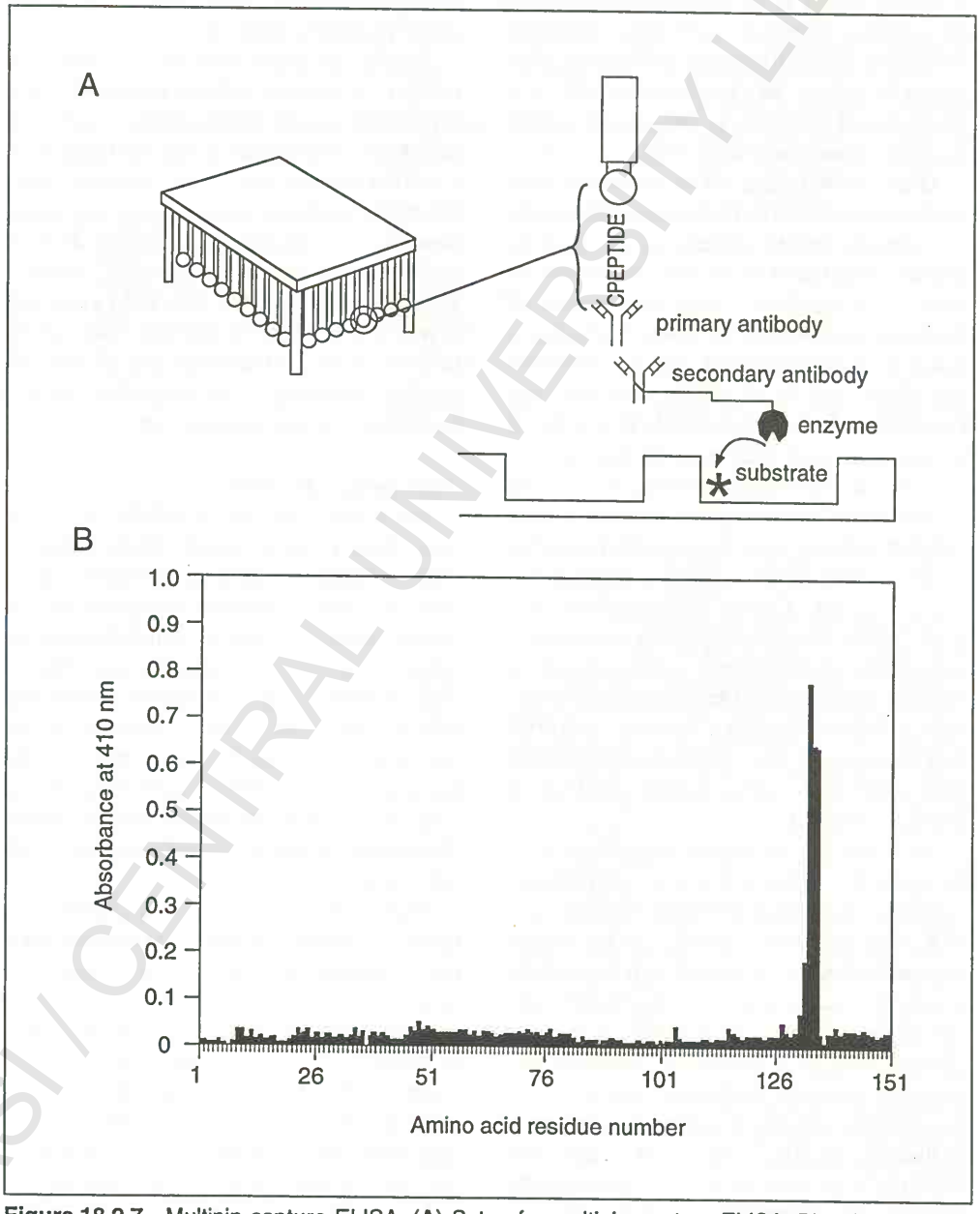

Figure 18.2.7 Multipin capture ELISA. (**A**) Setup for multipin capture ELISA. Pins (gears) with peptides covalently attached are incubated in primary antibody, secondary antibody, and substrate developer in ELISA plates. The absorbance is measured and the resulting absorbance values are graphed versus peptide number, corresponding to the N-terminal residue number of the peptide in the protein sequence. (**B**) Peptide pin capture ELISA results with a monoclonal antibody against pins bearing octamer peptides of gonococcal pilin protein. All the peptides that show high readings contain a significant portion of the epitope. (Diagram courtesy of Dr. Fred Cassels, Walter Reed Army Institute of Research, Washington, D.C.)

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thesis (MPS) pins have a great advantage over the noncleavable peptide (NCP) pin-bound peptides, as the latter can only be assayed once a day, whereas hundreds of parallel assays can be carried out on all biotinylated peptides at once. Reading data directly into a computer enables the massive amounts of data to be stored efficiently for later analysis.

Dispensing amino acids can be carried out efficiently by two people, one reading out the position into which the amino acid is to be dispensed and the other doing the actual dispensing. The passive partner (reader) can also act as a cross-checker to ensure no mistakes are made. If a computer-controlled pointing device is used, accuracy is improved and dispensing becomes a one-person operation. For large syntheses (>200 peptides), it is important that the dispensing be fast and accurate so that three couplings can be carried out per day.

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Synthetic Peptides for Production of Antibodies that Recognize Intact Proteins

Antibodies that recognize intact proteins can be produced through the use of synthetic peptides based on short stretches of the protein sequence, without first having to isolate the protein. The procedure for selecting stretches of protein sequence likely to be antigenic is relatively straightforward. However, no procedure will identify a single sequence guaranteed to be effective, nor will it usually identify the best single sequence to use. Rather, several sequences will be identified that have a higher-than-average probability of producing an effective antigen.

The steps to produce an effective antibody include: (1) designing the peptide sequence based on the sequence of the protein; (2) synthesizing the peptide; (3) preparing the immunogen either by coupling the synthetic peptide to a carrier protein or through the use of a multiple antigenic peptide (MAP); (4) immunizing the host animal; (5) assaying antibody titer in the host animal's serum; and (6) obtaining the antiserum and/or isolating the antibody. This unit covers steps 1 and 3; step 2 requires a laboratory with expertise in peptide synthesis. Peptide synthesis services are widely available both academically and commercially.

The best method to select potentially effective sequences is via a computer-assisted strategy (see Basic Protocol 1). An alternative manual method is also described (see Alternate Protocol 1) but is not recommended to replace the use of algorithms if there is a choice. A small synthetic peptide is usually insufficiently immunogenic on its own, and two methods have been developed to solve this problem. The first (see Basic Protocol 2) involves chemically coupling the synthetic peptide to a carrier protein to boost the immune response. The second method (see Alternate Protocol 2) entails direct synthesis of a MAP covalent multimer of the simple peptide sequence. Both methods have proven effective and it is a matter of personal preference which to use. Coupling to a carrier protein requires additional chemical manipulations after synthesis of the peptide, while the MAP is complete and ready for immunization at the conclusion of the synthetic protocol. Disadvantages of MAPs are that they are more difficult to produce homogeneously and to analyze postsynthetically. They also may be more prone to insolubility problems.

A carrier protein is a relatively large molecule capable of stimulating an immune response independently. A synthetic peptide coupled to a carrier protein acts as a hapten and produces antibodies specific for the hapten (antibodies against the carrier protein are also produced). The most commonly used carrier proteins are keyhole limpet hemocyanin (KLH) and bovine or rabbit serum albumin (BSA or RSA). KLH is usually preferred, because it tends to elicit a stronger immune response and is evolutionarily more remote from mammalian proteins. A common problem with KLH, however, has been its solubility. Pierce Chemical Company sells a preparation of KLH purported to have better solubility properties (see below).

Alternatively, peptides can be coupled to carrier proteins through either their amino (see Alternate Protocol 3) or carboxyl groups (see Alternate Protocol 4). These two alternate protocols are not recommended as a first choice for coupling, but are included because they have been used successfully and may be advantageous for certain special applications discussed in the Commentary. Also presented are methods for assaying free sulfhydryl content and for reducing disulfide bonds in synthetic peptides (see Support Protocols 1 and 2).

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Once the coupling procedure has been performed, it is possible to determine the approximate degree of coupling by amino acid analysis. However, in most instances this is unnecessary and the product can be used directly.

BASIC PROTOCOL 1

COMPUTER-ASSISTED SELECTION OF APPROPRIATE ANTIGENIC PEPTIDE SEQUENCES

An antibody produced in response to a simple linear peptide will most likely recognize a linear epitope in a protein. Furthermore, that epitope must be solvent-exposed to be accessible to the antibody. The general features of protein structure that correspond to these criteria are turns or loop structures, which are generally found on the protein surface connecting other elements of secondary structure, and areas of high hydrophilicity, especially those containing charged residues. As a consequence, computer algorithms that predict protein hydrophilicity and tendency to form turns are very useful. Several analytic programs or algorithms that attempt to do this have been developed. Although the choice of method may rely on availability or personal preference, there tends to be a high level of agreement among them. As stated earlier, none of the methods will identify the one single sequence guaranteed to produce an effective antibody against any given protein. Rather, the methods will offer several good candidates, one or several of which can be used.

Many of these algorithms may already be available on a local computer system. They are included in many commercial software packages such as GCG (Genetics Computer Group; see *SUPPLIERS APPENDIX*). The ExPASy Web site of the University of Geneva offers free access to a variety of different programs over the Internet at *http://expasy.hcuge.ch/www/tools.html*.

The following protocol utilizes the hydropathy index developed by Kyte and Doolittle (1982) and the secondary structure prediction method for β turns developed by Chou and Fasman (1974) found in the tool "Protscale" at the ExPASy Internet address.

1. Using the selected algorithms, compute the hydropathy index and the tendency for β-turns of the protein sequence. Use a window size of 7 or 9 and give equal weight to each amino acid. Record the results in either graphical or numerical form, or both.

   As an example, the graphical representation of these results for the protein sequence shown in Figure 18.3.1 is presented in Figure 18.3.2.

   A window size determines the number of amino acids to be used in computing a value for the amino acid at the center of the window. For example, a window size of 9 includes 4

```
      10         20         30         40         50         60
MAKVSLEKDK IKFLLVEGVH QKALESLRAA GYTNIEFHKG ALDDEQLKES IRDAHFIGLR
SRTHLTEDVI NAAEKLVAIG CFCIGTNQVD LDAAAKRGIP VFNAPFSNTR SVAELVIGEL 120
LLLLRGVPEA NAKAHRGVWN KLAAGSFEAR GKKLGIIGYG HIGTQLGILA ESLGMYVYFY 180
DIENKLPLGN ATQVQHLSDL LNMSDVVSLH VPENPSTKNM MGAKEISLMK PGSLLINASR 240
GTVVDIPALC DALASKHLAG AAIDVFPTEP ATNSDPFTSP LCEFDNVLLT PHIGGSTQEA 300
QENIGLEVAG KLIKYSDNGS TLSAVNFPEV SLPLHGGRRL MHIHENRPGV LTALNKIFAE 360
QGVNIAAQYL QTSAQMGYVV IDIEADEDVA EKALQAMKAI PGTIRARLLY
```

Figure 18.3.1 The amino acid sequence of a 410-residue protein analyzed by the method presented in Basic Protocol 1. The results are shown in Figure 18.3.2.

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amino acids on each side of the central amino acid. The value computed for the central amino acid is the simple average of the values for each amino acid in the window.

2. Compare the results of the two analyses and look for areas of sequence that are high in turn tendency and high in hydrophilicity (low in hydrophobicity).

   In Figure 18.3.2, these areas correspond to positive peaks in the Chou-Fasman analysis and negative peaks in the Kyte-Doolittle analysis. The three best areas in terms of amplitude and correlation are shaded. These correspond to the sequences underlined in Figure 18.3.1. (Note the alignment of these peak optima as compared to the peaks around residue 300.)

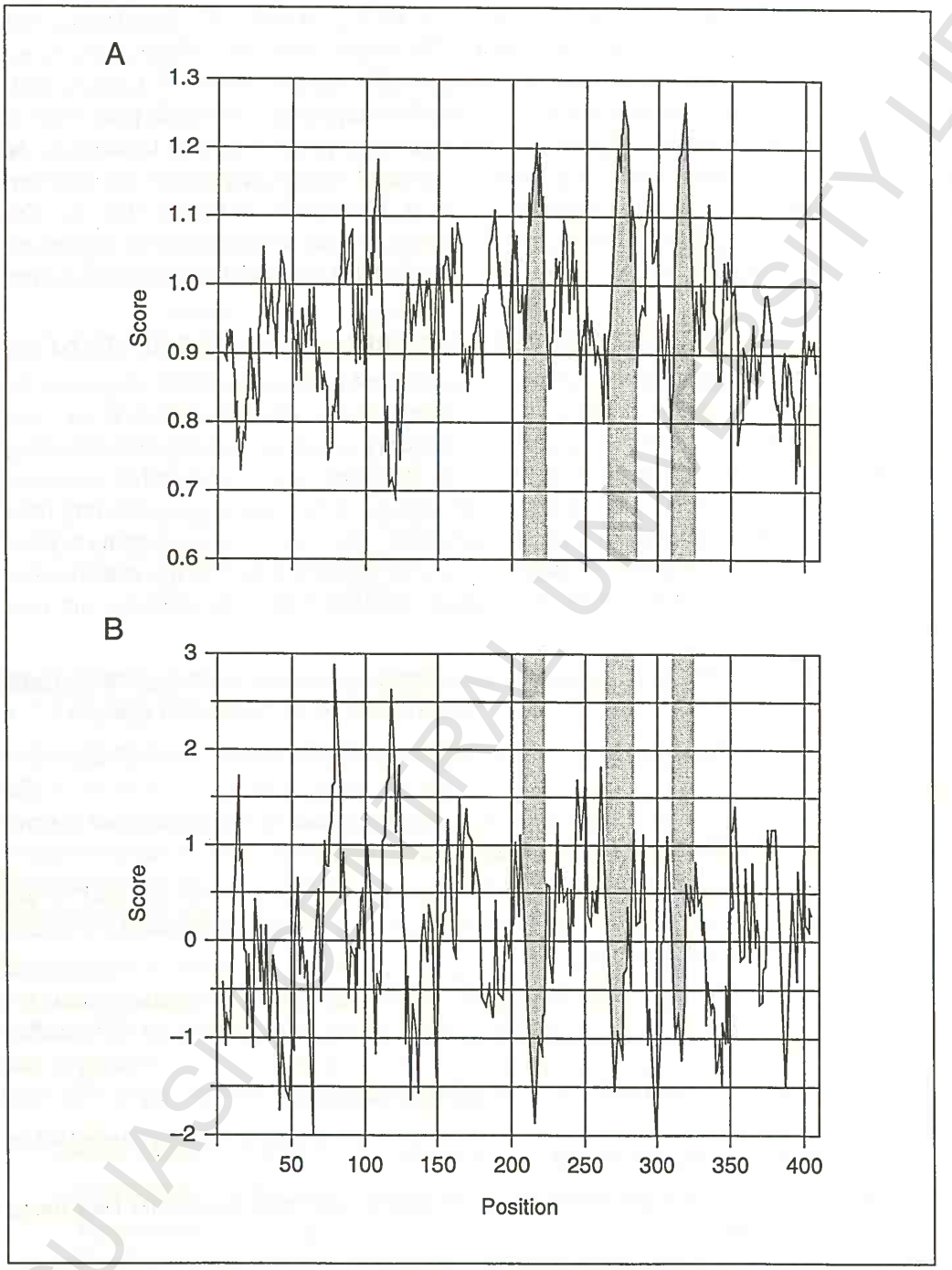

Figure 18.3.2 Graphical representation of the results generated by a computer algorithm for the sequence in Figure 18.3.1, analyzed by the method presented in Basic Protocol 1. The shaded areas represent three regions in the sequence meeting criteria for selection as potential immunogens. (**A**) Analysis for β turns (Chou and Fasman, 1974). (**B**) Analysis for hydrophobicity (Kyte and Doolittle, 1982).

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3. Examine the sequences for glycosylation site motifs and discard any sequences that contain them unless it is known that the protein is not glycosylated.

   Amino acids in glycosylated regions may be shielded from presentation to an antibody by masking carbohydrates.

   Amino-linked carbohydrate chains can occur at Asn-X-Ser or Asn-X-Thr sequences. Hydroxyl-linked carbohydrate chains do not appear to have a set motif.

4. Select the best sequences resulting from this analysis to use as antigenic peptides. These are sequences where the largest positive values (peaks with positive deflection) for turn propensity correspond in position to the largest negative values (peaks with negative deflection) for hydrophobicity. The values obtained in these analyses are relative and dependent on the individual protein's composition, so it is not possible to set an arbitrary minimum value as a cutoff for rejecting a particular peak. Rather, always select the peaks of greatest magnitude in any given sequence. In addition, the immediate amino-terminal and carboxyl-terminal regions of proteins are often exposed to solvent. If these areas appear to be hydrophilic in nature, they are also acceptable candidates. Thus each analysis may provide several potential sequences. How many peptides to make (see Anticipated Results) is a matter of individual choice.

ALTERNATE PROTOCOL 1

MANUAL INSPECTION TO SELECT APPROPRIATE PEPTIDE SEQUENCES

If computer algorithms are not available, it is possible to select potential sequences by manual inspection. Although there is no evidence that a manual method is any less effective than the use of computer algorithms, there is a greater probability of overlooking potentially important areas of sequence. It is therefore recommended that computer analysis be used whenever it is available. Although it can be done, it would be very time consuming and labor intensive to manually calculate values for every overlapping peptide offset by a single amino acid in the same way that the algorithms do. For this reason, areas rich in polar residues are selected for manual calculation of hydrophilicity and turn propensity.

1. Visually inspect the protein sequence and select areas that contain at least two to three charged residues (Lys, Arg, His, Asp, Glu) within a 10- to 15-residue span.

   If this criterion cannot be met, select sequences with the greatest number of charged residues.

2. From the sequences identified in step 1, select a subset of sequences that are the highest in Ser, Thr, Asn, Gln, Pro, and Tyr content.

3. Calculate average hydrophilicity and turn propensity for each amino acid in the selected sequences using the values given in Table 18.3.1 and a window of 9 residues (see Basic Protocol 1, step 1).

   Be sure to include the residues flanking the selected sequence for calculation of values for the residues at the ends of the selected sequence. In other words, don't use different size windows.

4. Plot the values for each amino acid of a chosen sequence.

   Sequences whose optimal values for hydrophilicity and turn propensity correspond (as in Fig. 18.3.2) are considered good candidates.

5. Inspect sequences for glycosylation motifs and discard these candidates (see Basic Protocol 1, step 3).

   Amino acids of glycosylated regions may be masked in native proteins, so an antibody raised against them would be ineffective.

6. Select the best sequences (see Basic Protocol 1, step 4 for criteria), choosing a high turn-propensity-to-hydrophobicity ratio.

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Table 18.3.1 Hydrophobic and β-Turn Indices of Amino Acids

Amino acid	Symbols	Hydrophobicity value[a]	β-turn propensity[b]
Arginine	Arg (R)	–4.5	0.95
Lysine	Lys (K)	–3.9	1.01
Aspartic acid	Asp (D)	–3.5	1.46
Glutamic acid	Glu (E)	–3.5	0.74
Asparagine	Asn (N)	–3.5	1.56
Glutamine	Gln (Q)	–3.5	0.98
Histidine	His (H)	–3.2	0.95
Proline	Pro (P)	–1.6	1.52
Tyrosine	Tyr (Y)	–1.3	1.14
Tryptophan	Trp (W)	–0.9	0.96
Serine	Ser (S)	–0.8	1.43
Threonine	Thr (T)	–0.7	0.96
Glycine	Gly (G)	–0.4	1.56
Alanine	Ala (A)	1.8	0.66
Methionine	Met (M)	1.9	0.60
Cysteine	Cys (C)	2.5	1.19
Phenylalanine	Phe (F)	2.8	0.60
Leucine	Leu (L)	3.8	0.59
Valine	Val (V)	4.2	0.50
Isoleucine	Ile (I)	4.5	0.47

[a]Kyte and Doolittle (1982).

[b]Chou and Fasman (1974).

DESIGNING A SYNTHETIC PEPTIDE FOR COUPLING TO A CARRIER PROTEIN

BASIC PROTOCOL 2

Although there is no direct evidence to show that the state of the termini of the peptide affects its ability to produce antibodies that will react with the protein, most procedures suggest that the termini of the peptide should mimic their native state. Thus, sequences whose terminal residues normally are in peptide linkage in the protein can have their amino-terminal and carboxyl-terminal groups modified by acetylation and amidation, respectively, during synthesis.

Modification of the amino or carboxyl termini will decrease the polarity of the peptide in solution and could have a significant effect on the peptide's solubility. If the peptide lacks sufficient protonatable side chains, modification of the termini can be omitted. A general rule to predict solubility is that the total number of charges at a given pH should be at least 20% of the number of residues in the peptide.

1. Choose a sequence of 10 to 15 amino acid residues for the synthetic peptide.

   Longer peptides are more difficult and expensive to make, and they are usually unnecessary.

   Try to choose a stretch of sequence that contains some charged residues such as Arg, Lys, His, Glu, and Asp. In addition to the high likelihood of these amino acids to be located on the surface of the protein, they aid handling of the synthetic product by promoting solubility.

2a. *If the selected sequence does not contain an internal cysteine:* Place a cysteine on either the amino or carboxyl terminus for use in coupling to a carrier protein with a

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heterobifunctional cross-linking reagent such as MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (see Basic Protocol 3).

Cross-linking with heterobifunctional reagents is the recommended procedure for most peptides (see Basic Protocol 3). As an alternative to using a chemical cross-linking reagent, any peptide, regardless of amino acid content, can also be photochemically linked to a carrier protein if p-benzoyl benzoic acid is added to the peptide during synthesis (see Alternate Protocol 5). Although photochemical cross-linking is effective, it is not widely used.

If the sequence includes the immediate amino or carboxyl terminal sequence of the protein, the cysteine should be placed on the end that would normally be engaged in the internal peptide bond. For sequences internal to the protein, the cysteine may be placed at either end according to the preference of the synthetic chemist. However, if amino-terminal capping (acetylation) is used after the coupling of each amino acid during synthesis, it is preferable to place the cysteine on the amino-terminal end of the peptide since then only the full-length peptide will contain the cysteine residue. In this way, if synthetic difficulties are encountered, only the full-length peptide will couple to the carrier. If placed on the carboxyl terminal end, the cysteine residue tends to racemize during synthesis unless a chlorotrityl resin is used. However, this should not have an effect on the rest of the peptide or the generation of antibodies.

2b. *If the sequence contains an internal cysteine residue:* Do not add a terminal cysteine for MBS cross-linking. Rather, use an alternative coupling procedure (see Alternate Protocols 3, 4, or 5) or synthesize a multiple antigenic peptide (MAP; see Alternate Protocol 2).

Internal cysteine sulfhydryl groups will also cross-link to the carrier protein and result in a peptide attached at multiple points. If the sulfhydryl will eventually be important for antibody recognition of the protein, the immunization may not produce effective antibodies. Furthermore, the additional constraint produced by the existence of multiple points of coupling may affect the ultimate ability of the antibody to recognize the protein.

3a. *For sequences whose terminal residues are in peptide linkage within the protein:* If the peptide is coupled using a heterobifunctional reagent such as MBS (see Basic Protocol 3), modify the amino and carboxyl ends by acetylation and amidation, respectively, during the synthetic procedure. If coupling is performed with a homobifunctional reagent that reacts with amino groups such as glutaraldehyde (see Alternate Protocol 3) or by the photochemical method (see Alternate Protocol 5), only amidate the carboxyl terminus. If coupling is performed with EDC (see Alternate Protocol 4), only acetylate the amino terminus.

3b. *For sequences that are amino or carboxyl terminal to the protein:* If the sequence is the immediate amino or carboxyl terminal sequence of the protein and the peptide will be coupled with a heterobifunctional reagent such as MBS (see Basic Protocol 3), leave the end that is not in peptide linkage (and the end that does not contain the additional cysteine residue for MBS coupling) as the free amino or carboxyl group unless it is known that they are normally blocked.

3c. *For sequences that are immediately amino terminal:* If the sequence is the immediate amino terminal sequence of the protein and the peptide will be coupled with a homobifunctional reagent that reacts with amino groups such as glutaraldehyde (see Alternate Protocol 3) or photochemically (see Alternate Protocol 5), amidate the C-terminus. If the sequence is the immediate carboxyl terminal sequence of the protein and the peptide will be coupled with glutaraldehyde (see Alternate Protocol 3) or photochemically (see Alternate Protocol 5), leave both termini free.

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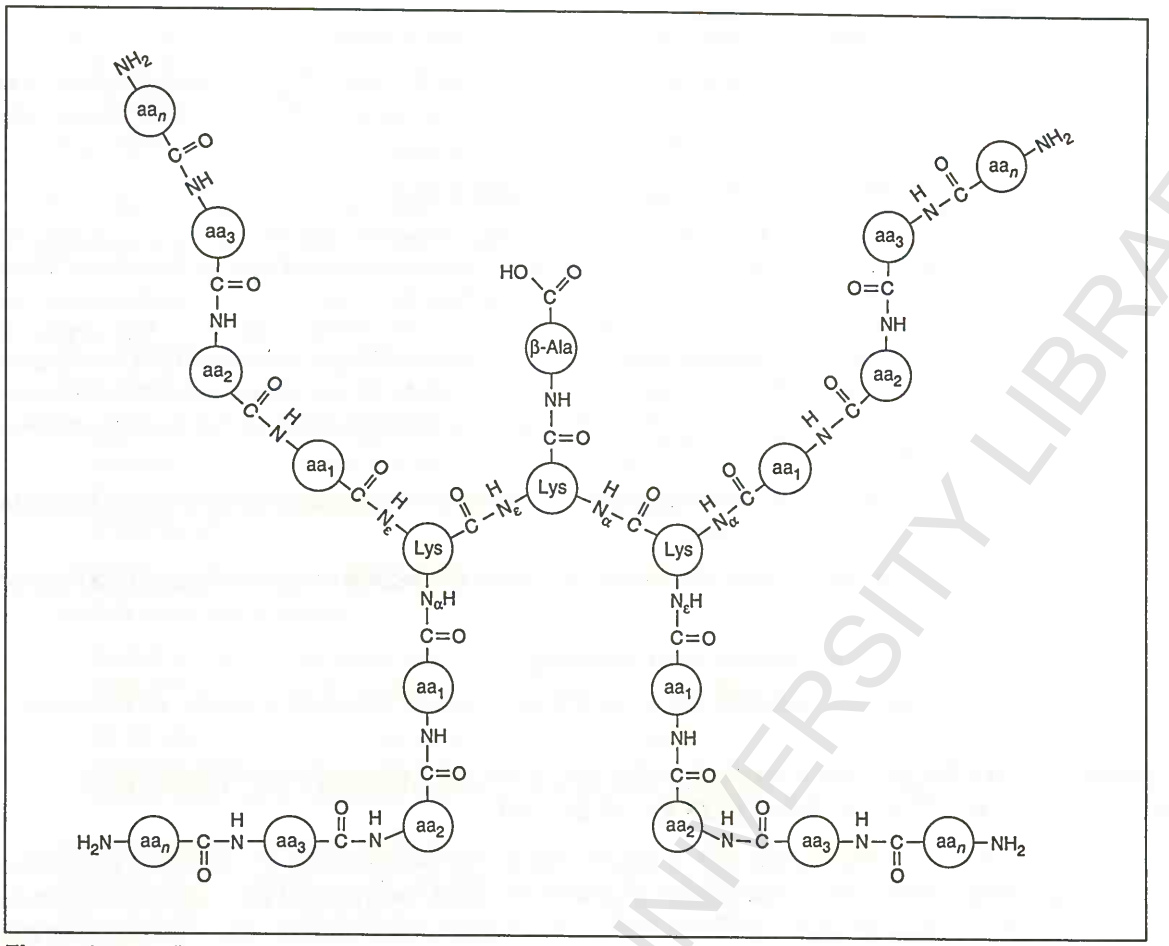

Figure 18.3.3 Representation of a four-branched multiple antigenic peptide (MAP). The MAP core is usually synthesized with a β-alanine residue attached to the solid phase support followed by a scaffold of lysine residues. The first lysine residue, attached through its α-carboxyl group to β-alanine, provides two primary amino groups (α and ε) for chain elongation. The next level of the lysine scaffold consists of two lysine residues which in turn provide four primary amino groups for chain elongation. MAPs with eight branches are formed by providing one additional layer of lysine to that depicted here.

4. If the peptide will be coupled with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; see Alternate Protocol 4), block the amino terminus by acetylation.

   This should be done even if the sequence is at the immediate amino terminus of the protein, since treatment with EDC can result in the production of covalent multimers of the peptide through reaction with the N-terminal amino group. Some procedures recommend using citraconylation to temporarily protect the amino group. However, this also adds a free carboxyl group that could result in multiple site attachment of the peptide to the carrier.

DESIGNING A SYNTHETIC MULTIPLE ANTIGENIC PEPTIDE

ALTERNATE PROTOCOL 2

A multiple antigenic peptide (MAP; Posnett et al., 1988; Tam, 1988) is an effective alternative to coupling a simple linear peptide to a carrier protein. MAPs are covalent constructs consisting of a simple peptide sequence synthesized on a branched core (Fig. 18.3.3) with one copy of the peptide sequence on each of four or eight branches. One advantage of a MAP is that it is suitable for use as an immunogen at the conclusion of the synthetic process. On occasion, MAPs have produced effective protein antibodies when the conventional peptide coupled to carrier protein has not. Thus, MAPs represent an effective alternative approach for antiserum production.

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1. Select a sequence between 10 and 15 residues in length.

   Longer sequences are unnecessary and increase the probability of synthetic problems. The presence of internal cysteine residues are not a concern with MAPs, but if present, take precautions to keep them reduced.

2. Synthesize the MAPs utilizing a four-branch core.

   Synthesize the MAP core de novo, or purchase resins for solid-phase peptide synthesis with four- or eight-branched cores (available commercially from Advanced ChemTech, Novabiochem, Perkin-Elmer, AnaSpec, and Bachem Bioscience). Eight-branched cores are suitable if the peptide is no more than 12 to 14 residues and has a high degree of hydrophilicity. There are more synthetic problems with eight-branched MAPs, presumably due to the higher density of structure during synthesis: they are more difficult to characterize and probably raise a diverse antibody population against some synthetic artifacts (see Mints et al., 1997).

3. *Optional.* If the selected sequence was not the amino terminus of the protein, acetylate the new amino terminus.

   In the case of a MAP, the carboxyl terminus will remain in covalent linkage to the branched core.

4. Use the MAP directly as an immunogen.

   Coupling to a carrier protein as described in Basic Protocol 3 is usually not necessary.

BASIC PROTOCOL 3

COUPLING SYNTHETIC PEPTIDES TO A CARRIER PROTEIN USING A HETEROBIFUNCTIONAL REAGENT

If the synthetic peptide was designed with a cysteine residue at one terminus (see Basic Protocol 2, step 2a), the following procedure should be followed for coupling to keyhole limpet hemocyanin (KLH) or other carrier proteins. Care must be taken to assure that the cysteine sulfhydryl group has remained reduced. Under normal synthetic conditions, if the peptide was lyophilized and stored dry immediately after synthesis, the sulfhydryl usually remains in the reduced state. The presence of free sulfhydryl groups in the peptide can be determined with Ellman's reagent (see Support Protocol 1) just prior to use; alternatively, high-resolution mass spectrometry can be used. If reduction is needed, follow the cysteine reduction procedure (see Support Protocol 2) before starting the coupling process.

The reagent most commonly used for this purpose is *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS). However, several related reagents (all available from Pierce) offer some additional features. Sulfo-MBS (Pierce) is a water-soluble alternative to MBS. Succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) and its sulfonated analog sulfo-SMCC provide the same chemistry with a more pH-stable maleimide (see step 1). The MBS and SMCC reagents can be used interchangeably in this protocol (the sulfo reagents can be dissolved in aqueous solution, while the others must be dissolved in an organic solvent; the concentrations listed are appropriate for all four).

Materials

Keyhole limpet hemocyanin (KLH; Pierce, Sigma, Calbiochem, or Boehringer Mannheim)
0.01 M sodium phosphate buffer, pH 7.5 (*APPENDIX 2E*)
10 mg/ml MBS in fresh *N*,*N*-dimethylformamide (DMF)
0.05 M and 0.1 M sodium phosphate buffer, pH 7.0 (*APPENDIX 2E*)
Synthetic peptide with a reduced cysteine residue at either the N- or C-terminus
6 M guanidine·HCl (see recipe)

Small glass vial with flat bottom

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~0.9 × 15–cm gel filtration column with Sephadex G-25 or G-50 (Pharmacia Biotech) or Bio-Gel P2 or P4 (Bio-Rad) resin; *or* prepacked PD-10 column (Pharmacia Biotech)

Additional reagents and equipment for gel filtration chromatography (*UNIT 8.3*)

NOTE: Do not use Tris or other buffers with primary amino groups in this procedure.

CAUTION: MBS is a moisture-sensitive irritant. Read the Material Safety Data Sheet before use.

1. Dissolve 5 mg KLH in ~0.5 ml of 0.01 M sodium phosphate buffer, pH 7.5, in a small, flat-bottomed vial.

   A pH range of 7.0 to 7.5 offsets competing reactions. Although the unprotonated form of the amine reacts with the N-hydroxysuccinimide ester and would be optimal at pH >8.0, hydrolysis of the ester bond and reaction of the maleimide group with amines is enhanced at higher pH.

2. Add 100 µl of 10 mg/ml MBS/DMF solution and stir gently with a micro stir-bar 30 min at room temperature.

   A small amount of precipitate may form during this procedure and is acceptable. However, if the precipitate is large, perform the procedure again with fresh components.

   As an alternative to performing this coupling procedure from scratch, it is possible to purchase MBS-activated KLH (Pierce; Boehringer Mannheim) or kits containing MBS-activated KLH and an alternate MBS-activated protein for use in ELISA assays (Pierce).

3. Separate MBS-activated KLH from free MBS on a ~0.9 × 15–cm gel filtration column, equilibrating and eluting the column with 0.05 M sodium phosphate buffer, pH 7.0. Collect 0.5-ml fractions and read their absorbance at 280 nm (*UNIT 8.3*).

   The first peak to elute is the KLH-MBS conjugate. These fractions may appear cloudy. The second peak is uncoupled MBS.

4. Pool the KLH-MBS conjugate fractions in a separate tube.

5. Dissolve 5 mg of the synthetic peptide in 0.01 M sodium phosphate buffer, pH 7.0, immediately prior to use. If the peptide is poorly soluble, use 6 M guanidine·HCl.

   Maleimide groups react specifically with sulfhydryls at slightly acid to neutral pH.

6. Add the peptide solution to the KLH-MBS conjugate. Stir gently with a micro stir-bar 3 hr at room temperature.

   The coupling may be continued overnight.

7. Dialyze against 4 liters distilled water overnight at 4°C. Use for immunizations within 24 hr.

ASSAY OF FREE SULFHYDRYLS WITH ELLMAN'S REAGENT

SUPPORT PROTOCOL 1

Free sulfhydryl content of a peptide can be quantitatively determined with Ellman's reagent, 5,5′-dithio-bis(2-nitrobenzoic acid). The molar extinction coefficient at 412 nm of thionitrobenzoate, the colored species generated when the reagent reacts with a free thiol, is 14,150 in 0.1 M sodium phosphate buffer (see Fig. 18.3.4). The sensitivity of the reaction is in the low nmol/ml range for sulfhydryl groups, making it well suited for synthetic peptides. By dry weight most synthetic peptides are only ~60% to 75% peptide, the remainder consisting of counterions and water of hydration. Amino acid analysis (see *UNIT 11.9*) is needed to establish the actual peptide content unambiguously, but such precise measurement is not usually necessary for qualitative evaluation of the free sulfhydryl content of a peptide sample.

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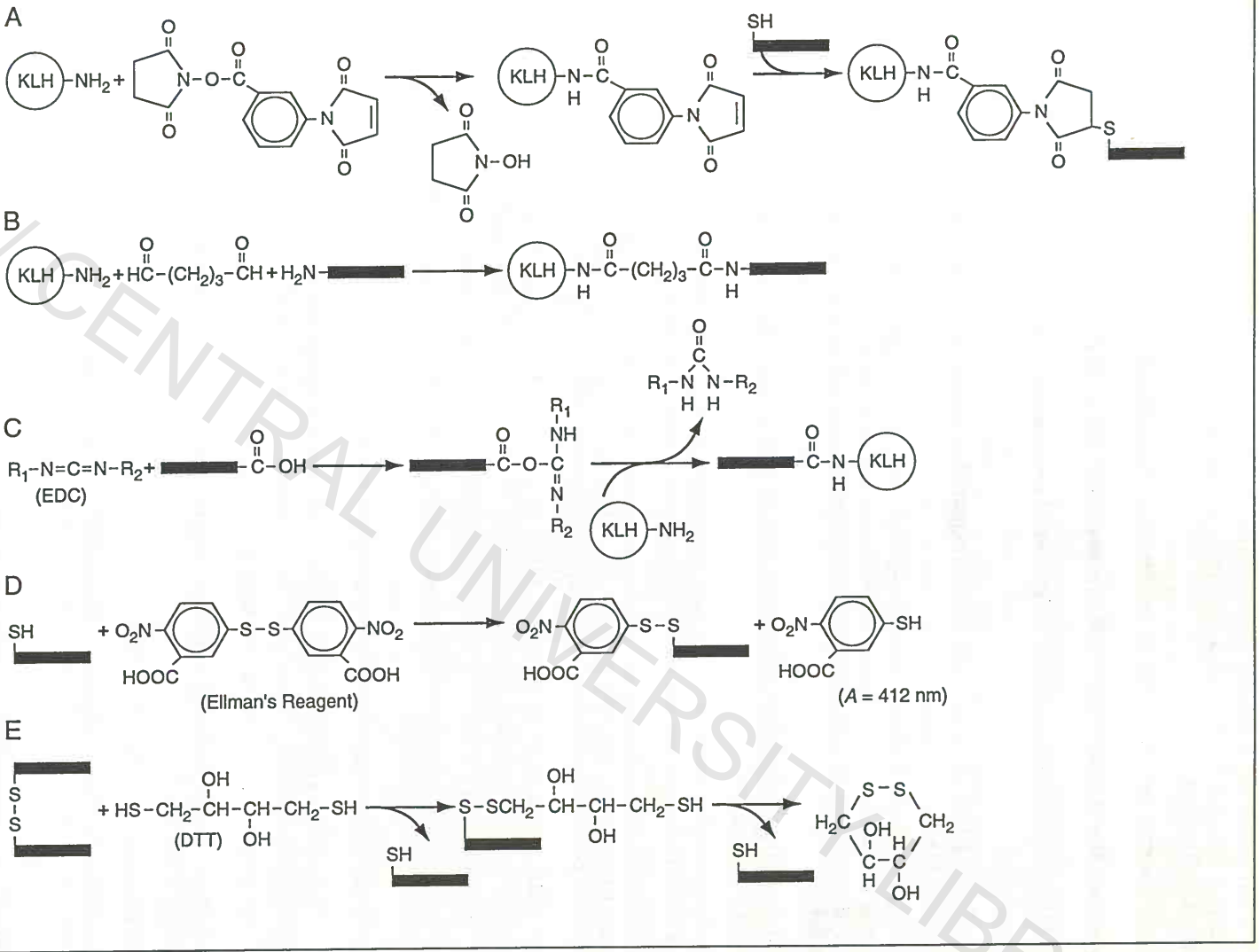

Figure 18.3.4 Representations of the chemistries described in this unit. (**A**) Cross-linking with MBS; (**B**) cross-linking with glutaraldehyde; (**C**) cross-linking with EDC; (**D**) reaction of free sulfhydryls with Ellman's reagent to produce a colorimetric product; (**E**) reduction of disulfides with DTT. Abbreviations: KLH, keyhole limpet hemocyanin; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; EDC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide; DTT, *N,N*-dithiothreitol.

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Materials

Cysteine standard stock solution (see recipe)
0.1 M sodium phosphate, pH 8.0 (*APPENDIX 2E*)
Peptide to be assayed
Ellman's reagent solution (see recipe)
13 × 100–mm glass test tubes

1. Prepare a cysteine standard curve by adding 25 µl, 50 µl, 100 µl, 150 µl, 200 µl, and 250 µl of cysteine standard stock solution to separate 13 × 100–mm tubes. Add ≤250 µl of each peptide to be tested to separate tubes. Bring the volume in each tube to 250 µl with 0.1 M sodium phosphate, pH 8.0. Add 250 µl of 0.1 M sodium phosphate, pH 8.0, to a blank tube.

   The cysteine content of the peptide to be assayed should fall within the range of the standard curve (37.5 to 375 nmol).

2. Add 50 µl Ellman's reagent solution and 2.5 ml of 0.1 M sodium phosphate, pH 8.0, to each tube. Mix and incubate 15 min at room temperature.
3. Measure absorbance at 412 nm (A_{412}).
4. Plot the A_{412} values of the standards after subtracting the value for the blank to produce a standard curve. Use this curve to determine the free sulfhydryl content of the peptides.

REDUCING CYSTEINE GROUPS IN PEPTIDES

SUPPORT PROTOCOL 2

When a peptide is synthesized with a terminal cysteine residue to be used for coupling with MBS (see Basic Protocol 3), the cysteine must be in the reduced state (present as free -SH rather than as a disulfide) in order to participate in the reaction with the coupling reagent. If peptides are lyophilized immediately after extraction from the resin cleavage cocktail or reversed-phase HPLC and used immediately after reconstitution, oxidation of cysteine side chains is usually not a problem. However, if oxidation to disulfides has occurred, the peptide can be reduced prior to use with the protocol presented here.

Dithiothreitol (DTT) is preferred to 2-mercaptoethanol (2-ME) as a reducing agent because its lower redox potential allows it to be effective at lower concentrations, and the reaction goes to completion because formation of the six-membered ring containing an internal disulfide is energetically favorable (see Fig. 18.3.4; also see *APPENDIX 3A*).

To determine if reduction is necessary, quantitate the level of free sulfhydryl groups with Ellman's reagent (see Support Protocol 1).

Materials

Synthetic peptide
0.1 M sodium phosphate, pH 8.0 (*APPENDIX 2E*)
1 M aqueous dithiothreitol (DTT)
1 N HCl

100- or 250-µl polypropylene tubes
Nitrogen gas source

Additional reagents and equipment for reversed-phase HPLC of peptides (see *UNIT 11.6*)

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1. Dissolve 5 to 10 mg of peptide in 0.1 M sodium phosphate, pH 8.0.
2. Add 100 µl of 1 M DTT.
3. Flush nitrogen over the surface of the liquid, seal the tube, and incubate 1 hr at 37°C.
4. Acidify with 1 N HCl and desalt by reversed-phase HPLC (*UNIT 11.6*).
5. Pool peptide fractions and lyophilize. Store lyophilized at 4°C until ready to use (up to several days).

   The oxidation state of the peptide can usually be followed by analytical monitoring of its elution position on reversed-phase HPLC. Disulfide-linked dimers of peptides generally elute later than the monomeric peptide.

ALTERNATE PROTOCOL 3

COUPLING SYNTHETIC PEPTIDES TO A CARRIER PROTEIN USING A HOMOBIFUNCTIONAL REAGENT

The available homobifunctional reagents couple compounds through primary amino groups. Therefore, peptides with internal lysine residues should not be used in this procedure. The reagent most commonly used for this procedure is glutaraldehyde, but it should not be used with peptides containing internal Cys, Tyr, or His residues. Other homobifunctional cross-linking reagents that can be used in the same way as glutaraldehyde, but do not cross react with Cys, Tyr, or His residues, are also available: disuccinimidyl suberate (DSS), disuccinimidyl glutarate (DSG), and bis (sulfosuccinimidyl) suberate (BS3; all available from Pierce). However, these reagents are not widely employed for coupling peptides to proteins and are not considered the method of choice, and methods for their use have not been formalized. This is probably because coupling of the synthetic peptide to itself and aggregation of the carrier protein can occur with homobifunctional reagents such as these. Glutaraldehyde, on the other hand, although also subject to this limitation, has generally been used successfully.

Additional Materials (also see Basic Protocol 3)

50 mM sodium borate buffer, pH 8.0 (pH adjusted with HCl)
Glutaraldehyde solution (see recipe)
1 M glycine in 50 mM sodium borate buffer, pH 8.0

NOTE: Do not use Tris or other buffers with primary amino groups in this procedure.

1. Dissolve 5 mg KLH in ~1.0 ml of 50 mM sodium borate buffer, pH 8.0.
2. Add 5 mg synthetic peptide.
3. Slowly add 1 ml fresh glutaraldehyde solution with gentle mixing at room temperature. Allow to react for an additional 2 hr with gentle mixing.

   Formation of a yellowish color or milkiness is normal and does not affect the sample.

4. Add 0.25 ml of 1 M glycine to bind unreacted glutaraldehyde.

   A darker yellow to brown color may develop.

5. Dialyze the reaction mixture overnight at 4°C against 4 liters of 50 mM sodium borate buffer, pH 8.0, and then overnight against water. Use immediately.

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COUPLING SYNTHETIC PEPTIDES TO A CARRIER PROTEIN USING A CARBODIIMIDE

ALTERNATE PROTOCOL 4

This procedure couples amino groups to carboxyl groups by way of activation of the carboxyl group with a water-soluble carbodiimide. Since the procedure is most easily performed in one step, peptides containing internal Asp, Glu, Lys, Tyr, or Cys residues should not be used. Also, in order to avoid making polymers of the peptide, the amino terminus should be blocked by acetylation during synthesis (see Basic Protocol 2). While this method has been used successfully, it is not considered to be the method of choice except in special situations.

Additional Materials (also see Basic Protocol 3)

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Pierce), used fresh or stored desiccated and frozen
0.1 N HCl

NOTE: Buffers containing amino or carboxyl groups should not be used in this procedure. According to some reports, buffers containing phosphate groups should also be avoided. Water is the safest choice as a solvent.

1. Dissolve 5 mg synthetic peptide in 1 ml water.
2. Add 25 mg EDC and carefully adjust pH to 4.0 to 5.0 by adding small amounts of 0.1 N HCl. Allow to react for 5 to 10 min at room temperature with gentle mixing.

   pH paper suffices to monitor this adjustment.
3. Dissolve 5 mg KLH in 0.5 ml water and add to solution from step 2. React 2 hr at room temperature with gentle mixing.
4. Dialyze against 4 liters of water overnight at 4°C. Use immediately.

COUPLING SYNTHETIC PEPTIDES TO A CARRIER PROTEIN PHOTOCHEMICALLY

ALTERNATE PROTOCOL 5

Most synthetic peptides today are made with 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry (see Fig. 18.1.1), and therefore the following protocol links the photoactive group to the free amino terminus of the peptide. An alternative approach if *t*-butyloxycarbonyl (Boc) chemistry is used is to link it to an ε-amino group of a terminal lysine (Gorka et al., 1989).

Additional Materials (see also Basic Protocol 3)

4-benzoyl benzoic acid (Sigma or Aldrich)
Quartz spectrophotometry cuvettes

1. Have the synthetic chemist who is making the peptide attach a benzoyl benzoic acid group to the amino terminus simply by treating the reagent as the terminal residue during normal synthesis.

   This blocks the amino terminus.
2. Dissolve 5 mg KLH in ~0.5 ml of 0.01 M sodium phosphate buffer, pH 7.5.
3. Add 2 mg of synthetic peptide containing the benzoyl benzoate adduct.
4. Place in a 1-cm quartz cuvette and irradiate with 366-nm light for 3 hr at a distance of 0.5 cm. Use immediately.

   The peptide can be used directly for immunization.

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REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Cysteine standard stock solution

Dissolve 26.3 mg cysteine hydrochloride monohydrate in 100 ml of 0.1 M sodium phosphate, pH 8.0 (*APPENDIX 2E*). Prepare immediately before use.

Ellman's reagent solution

Dissolve 4 mg Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid) (Pierce), in 1 ml of 0.1 M sodium phosphate, pH 8.0. Prepare immediately before use.

Glutaraldehyde solution, 0.15%

Add 30 ml of 25% aqueous glutaraldehyde solution to 5 ml of 50 mM sodium borate buffer, pH 8.0 (pH adjusted with HCl). Prepare fresh and use immediately. If the glutaraldehyde precipitates, check the pH. It should not be above 8.0; a slightly lower pH can be used (pH 7 to 8).

CAUTION: *Glutaraldehyde is a sensitizing agent that should be handled in a hood and only according to the recommendations in the Material Safety Data Sheet. When mixing solutions or performing reactions, keep the container covered to prevent vapors from escaping into the atmosphere.*

Guanidine·HCl, 6 M

Dissolve 1 g guanidine·HCl in 1 ml of 0.05 M sodium phosphate, pH 7.0. Store up to several weeks at room temperature.

The resulting 1.8-ml solution should be ~0.025 M phosphate/6 M guanidine·HCl at pH 7.0.

COMMENTARY

Background Information

Synthetic peptides are linear arrays of amino acids that in most instances possess a random structure in solution. While it is not difficult to produce antipeptide antibodies, it does not necessarily follow that the antibodies will recognize a protein containing the same stretch of sequence found in the peptide. In order for this to occur, the amino acids in the protein must be oriented to the antibody in a way similar to that of the synthetic peptide. This generally requires three basic features of the protein: (1) that the stretch of sequence be exposed to solvent; (2) that the sequence be a continuous stretch of amino acids; and (3) that it not possess a higher-order structure that renders it unrecognizable by the antibody population.

The large number of model protein structures now available indicate that almost all of the ionized groups in water-soluble proteins are on the protein surface. Asp, Glu, Lys, and Arg residues, on the average, comprise 27% of the protein surface and only ~4% of the protein interior. The fraction of residues at least 95% buried range from 0.36 to 0.60 for nonpolar residues and 0.01 to 0.23 for polar residues. Only 1% of Arg and 3% of Lys residues fall into the 95% buried range (Creighton, 1993). Therefore, it is reasonable to expect solvent-exposed areas of proteins to display relatively high levels of polar and charged residues, particularly Arg and Lys.

Proteins display three kinds of secondary structure: α-helices, β-sheets, and turns or loops (see *UNIT 17.1*). Turns or loops generally connect elements of α-helices and β-sheets, and can either fit one of several rather strict motifs with recognizable hydrogen bonding patterns or be of a more extended, random nature. These turn or loop structures appear to be most useful for antibody production because they tend to be found on the surface of proteins connecting larger arrays of helices and sheets, and they consist of continuous stretches of amino acids. Although many amino acid residues in helices and sheets are also exposed at the surface, the regular geometry of amino acids contained within them makes them less suitable for this purpose. For instance, in β-sheet structures the side chain of each successive amino acid in the β-sheet strand points in the opposite direction to the ones immediately preceding and following it. Thus, even if the amino acid side chains are not predominantly buried in the interior of

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the protein, only every other side chain is exposed on the same surface of the sheet. This can hinder recognition by an antibody produced with a linear peptide capable of assuming a more random structure. A similar situation exists for α-helices. Although the change in direction of the side chains of successive amino acids is perhaps not as abrupt as in β-sheets, only approximately every third or fourth side chain is found on the same surface of the helix. Epitopes in proteins have been identified in amphipathic helices, but unless the synthetic peptide assumes a similar helical structure in solution, recognition by the antibody may be problematic.

These considerations have led to more useful methods for predicting sequences that will produce antibodies recognizing intact proteins. A variety of different indices that predict hydrophilicity or hydrophobicity and secondary structure are available (see *UNITS 2.2 & 2.3*, respectively). In addition, predictive methods based on segmental mobility, side chain accessibility, and sequence variability (see Van Regenmortel et al., 1988) have also been proposed. All of these methods generally tend to yield similar results, but it must be noted that these procedures were developed for (and work best with) water-soluble proteins composed of a single globular structure. Additional complications can arise with multisubunit proteins, where normally exposed structures may be shielded by subunit interactions, or membrane proteins with large sections shielded from the solvent.

The method presented in this unit utilizes the correlation between the hydrophilic character of a peptide sequence (Kyte and Doolittle, 1982) and its propensity to form β-turn structures (Chou and Fasman, 1974). Free access to these and many other algorithms is provided at the ExPASy Web site of the University of Geneva at *http://expasy.hcuge.ch/www/tools.html*.

After selection of the peptide sequence, an effective immunogen is generally produced by coupling the peptide to a carrier protein or by synthesizing a multiple antigenic peptide (MAP), with four or eight identical peptides assembled simultaneously on the α and ε amines of the terminal lysines of a branched core (see Fig. 18.3.3).

Critical Parameters

Analyzing protein sequences with algorithms or tables of assigned values for amino acids is a well-established procedure, but evaluating these results and selecting the candidate sequences requires some consideration. To take full advantage of the results, choose areas of sequence that give the maximum values for the properties being evaluated and that also show the highest degree of residue-by-residue correlation. In other words, choose areas of maximum amplitude where the centers of the peaks correspond to the same sequence with a divergence of no more than two to three residues. Examples of this are given in Figure 18.3.2, which shows results from the method presented in Basic Protocol 1 for the sequence shown in Figure 18.3.1. The top panel in Figure 18.3.2 predicts β-turns as calculated by the method of Chou and Fasman (1974). The bottom panel is a prediction of hydrophobicity using the parameters of Kyte and Doolittle (1982). The data are analyzed by looking for areas of high turn propensity (maximum positive deflection in the top panel) and high hydrophilicity (maximum negative deflection in the bottom panel). The shaded areas in Figure 18.3.2 designate three segments that meet these criteria. Note that the maximum and minimum values of these three stretches of protein sequence correlate very well. Additional areas of high hydrophilicity (bottom panel) are found near residues 64, 132, 137, 149, and 345, although the β-turn values of these secondary candidates are not as high as those of the three shaded areas. Two equally hydrophilic areas at residues 49 and 299 correspond to downward deflections in the β-turn profile and are thus not good candidates based on this analysis.

Many different chemistries are available for coupling synthetic peptides to carrier proteins to produce effective immunogens (Van Regenmortel et al., 1988). In many cases, however, side reactions or incompatibilities in chemistry between the coupling agent and the residues present in the peptide can be problematic. In order to simplify the process and present the greatest probability of success in most cases, only a few coupling methods are presented in this unit. In this regard, the recommended coupling procedure is cross-linking of the peptide via cysteine residues to keyhole limpet hemocyanin (KLH) with the heterobifunctional reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS; see Basic Protocol 3). This effective method has enjoyed great success and can be used for virtually any peptide. The one caveat is that it is not recommended for peptides with internal cysteine residues, since they will also link to the carrier. It is also critical, when coupling with MBS through an added terminal

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cysteine residue, that the sulfhydryl group of the peptide be present in the free or reduced form (see Support Protocols 1 and 2).

In addition to MBS coupling, other procedures commonly used (see Alternate Protocols 3 and 4) are included as alternatives for use in special situations, but these are not recommended as a general alternative to MBS because they are more restrictive and have the potential for undesirable side reactions. Glutaraldehyde coupling (see Alternate Protocol 3) should not be used with peptides containing internal Lys, Cys, Tyr, or His residues and, since it is a homobifunctional reagent, cross-linking of the peptide to itself and the carrier to itself can occur. The latter lowers antigenicity and can result in extensive aggregation and precipitation of the carrier. 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC; see Alternate Protocol 4) is a water-soluble carbodiimide and should not be used with peptides containing internal Lys, Glu, Asp, Tyr, or Cys residues. Alternate Protocol 5 describes a simple photochemical coupling strategy (Gorka et al., 1989).

Another good alternative for most peptides is the production of a multiple antigenic peptide (MAP; see Alternate Protocol 2). With this method the composition of the peptide is not a concern beyond its potential solubility properties. In most cases, since hydrophilic sequences are selected, this also is not a major problem. Both four- and eight-branched MAPs have been found to be effective. However, four-branched MAPs are recommended because they are less prone to synthesis problems and are easier to characterize.

As with any synthetic peptide, the product must be well characterized before use. If the peptide is not what it was intended to be, this decreases the probability of generating antibodies that will recognize the protein. At the very least, check synthetic peptides for homogeneity by analytical HPLC and correct mass by mass spectrometry (see Chapter 16). Characterization of MAP can be more problematic due to their multibranched nature (Mints et al., 1997): HPLC and mass spectrometric analysis can be compromised by the presence of four to eight peptide chains per molecule, each of which may have only a small percentage of modification at any particular residue but which in the aggregate contribute to broad spectra. However, this feature of MAPs usually does not tend to compromise their ability to form antigens of the proper peptide since the correct sequence is usually present in high enough concentration that a significant amount of specific antibody is produced among the polyclonal population. Amino acid analysis (*UNIT 11.9*), which is less sensitive to multiple small differences, tends to give a reasonable assessment of the MAP integrity.

Anticipated Results

The methods outlined in this unit produce an effective polyclonal antiserum against an intact protein from a single peptide sequence ~50% to 70% of the time. Therefore, it is advisable to prepare two or three different peptides from a given protein to increase the probability of at least one of them being effective.

Time Considerations

Computer-assisted analysis of a protein sequence and inspection of the data to select several candidate sequences takes from 5 to 30 min. Manual analysis of a protein sequence can take several hours but can certainly be accomplished in <1 day. Selection of peptide design and manner of synthesis as well as selection of a coupling method will take <1 hr. Actual preparation of the peptide can be accomplished in 3 to 4 days, but this may vary depending on the turnaround time of the synthetic laboratory. Coupling a synthetic peptide to a carrier protein takes from 1 to 2 days. Although not covered in this unit, production of the antisera will vary with the animal and protocol used, but generally requires 2 to 3 months. It is therefore advisable to inject several animals with different peptides at one time.

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Web site for programs to analyze protein sequences.

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St. Louis, Missouri

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Characteristics of Amino Acids

The physical properties of the amino acids determine the structure and function of the proteins in which they are found. Some useful details and relevant physical characteristics of the amino acids can be found in Table A.1A.1. Information about modified amino acids is provided in Table A.1A.2. The chemical structures of the amino acids, and an explanation of the role these structures play in enzymes, can be found in Figure A.1A.1. The three-dimensional structure of proteins is largely determined by the packing of their hydrophobic cores; the properties of amino acids that govern this packing are their relative hydrophobicities, which are discussed in *UNIT 2.2*, and their shapes and volumes, which can be assessed by referring to the space-filling models shown in Figure A.1A.2.

Post-translational modifications will change the mass of a protein or peptide; values for some common mass changes are listed in Table A.1A.3. The changes in mass resulting from modification of proteins with different carbohydrates are shown in Table A.1A.4.

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Table A.1A.1 Compositions and Masses of the Twenty Commonly Occurring Amino Acid Residues[a,b]

Name	Composition	Monoisotopic mass	Average mass	pK_a of ionizing side chain[c]	Occurrence in proteins (%)
Alanine (Ala, A)	C_3H_5NO	71.03711	71.0788	—	8.3
Arginine (Arg, R)	$C_6H_{12}N_4O$	156.10111	156.1876	~11.5–12.5 (12)	5.7
Asparagine (Asn, N)	$C_4H_6N_2O_2$	114.04293	114.1039	—	4.4
Aspartic acid (Asp, D)	$C_4H_5NO_3$	115.02694	115.0886	3.9–4.5 (4)	5.3
Cysteine (Cys, C)	C_3H_5NOS	103.00919	103.1448	8.3–9.5 (9)	1.7
Glutamic acid (Glu, E)	$C_5H_7NO_3$	129.04259	129.1155	4.3–4.5 (4.5)	6.2
Glutamine (Gln, Q)	$C_5H_8N_2O_2$	128.05858	128.1308	—	4.0
Glycine (Gly, G)	C_2H_3NO	57.02146	57.0520	—	7.2
Histidine (His, H)	$C_6H_7N_3O$	137.05891	137.1412	6.0–7.0 (6.3)	2.2
Isoleucine (Ile, I)	$C_6H_{11}NO$	113.08406	113.1595	—	5.2
Leucine (Leu, L)	$C_6H_{11}NO$	113.08406	113.1595	—	9.0
Lysine (Lys, K)	$C_6H_{12}N_2O$	128.09496	128.1742	10.4–11.1 (10.4)	5.7
Methionine (Met, M)	C_5H_9NOS	131.04049	131.1986	—	2.4
Phenylalanine (Phe, F)	C_9H_9NO	147.06841	147.1766	—	3.9
Proline (Pro, P)	C_5H_7NO	97.05276	97.1167	—	5.1
Serine (Ser, S)	$C_3H_5NO_2$	87.03203	87.0782	—	6.9
Threonine (Thr, T)	$C_4H_7NO_2$	101.04768	101.1051	—	5.8
Tryptophan (Trp, W)	$C_{11}H_{10}N_2O$	186.07931	186.2133	—	1.3
Tyrosine (Tyr, Y)	$C_9H_9NO_2$	163.06333	163.1760	9.7–10.1 (10.0)	3.2
Valine (Val, V)	C_5H_9NO	99.06841	99.1326	—	6.6

[a]For corresponding structures, see Figure A.1A.1 and Figure A.1A.2.

[b]The molecular mass of a normally terminated and unmodified peptide or protein may be calculated by summing the masses of the appropriate amino acid residues and adding the masses of H and OH for the N and C termini, respectively. In cases where cysteines are linked to form disulfide bridges, the mass of two hydrogen atoms should be subtracted for each disulfide bridge in the molecule. Specifically, monoisotopic masses were calculated using the atomic masses of the most abundant isotope of the elements: C = 12.0000000, H = 1.0078250, N = 14.0030740, O = 15.9949146, and S = 31.9720718. Average masses were calculated using the atomic weights of the elements: C = 12.011, H = 1.00794, N = 14.00674, O = 15.9994, and S = 32.066.

[c]These values are included for anyone wishing to make approximate isoelectric point determinations based on protein composition. Values for the terminal residues depend on the identity of the residue: α-amino, pK_a 6.8–8.2 (8.0); α-carboxyl, pK_a 3.2–4.3 (3.6). Values in parentheses are based on those given by Matthew et al. (1978) and provide a good starting point for determinations.

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Table A.1A.2 Compositions and Masses of Unusual and Modified Amino Acid Residues[a]

Name	Composition	Monoisotopic mass	Average mass
Carboxymethylcysteine (Cme)	$C_5H_7NO_3S$	161.01466	161.1815
Carboxyamidomethyl cysteine (Cam)	$C_5H_8N_2O_2S$	160.030650	160.195940
Pyridylethylcysteine (PECys)	$C_{10}H_{12}N_2OS$	208.06703	208.23239
Acrylamide-modified cysteine (AACys)	$C_6H_{10}N_2O_2S$	174.04631	174.22363
Cysteic acid [Cys(O_3H)]	$C_3H_5NO_4S$	150.99393	151.1430
Dehydroalanine (Dha)	C_3H_3NO	69.02146	69.0630
4-Carboxyglutamic acid (Gla)	C_4H_5NO	173.03242	173.1253
Homoserine (Hse)	$C_4H_7NO_2$	101.04768	101.1051
Hydroxylysine (Hyl)	$C_6H_{12}N_2O_2$	144.08988	144.1736
Hydroxyproline (Hyp)	$C_5H_7NO_2$	113.04768	113.1161
Isovaline (Iva)	C_5H_9NO	99.06841	99.1326
Norleucine (Nlc)	$C_6H_{11}NO$	113.08406	113.1595
Ornithine (Orn)	$C_5H_{10}N_2O$	114.07931	114.1473
Pyroglutamic acid (Pyr)	$C_5H_5NO_2$	111.03203	111.1002
Sarcosine (Sar)	C_3H_5NO	71.03711	71.0788

[a]Monoisotopic masses were calculated using the atomic masses of the most abundant isotope of the elements: C = 12.0000000, H = 1.0078250, N = 14.0030740, O = 15.9949146, and S = 31.9720718. Average masses were calculated using the atomic weights of the elements: C = 12.011, H = 1.00794, N = 14.00674, O = 15.9994, and S = 32.066.

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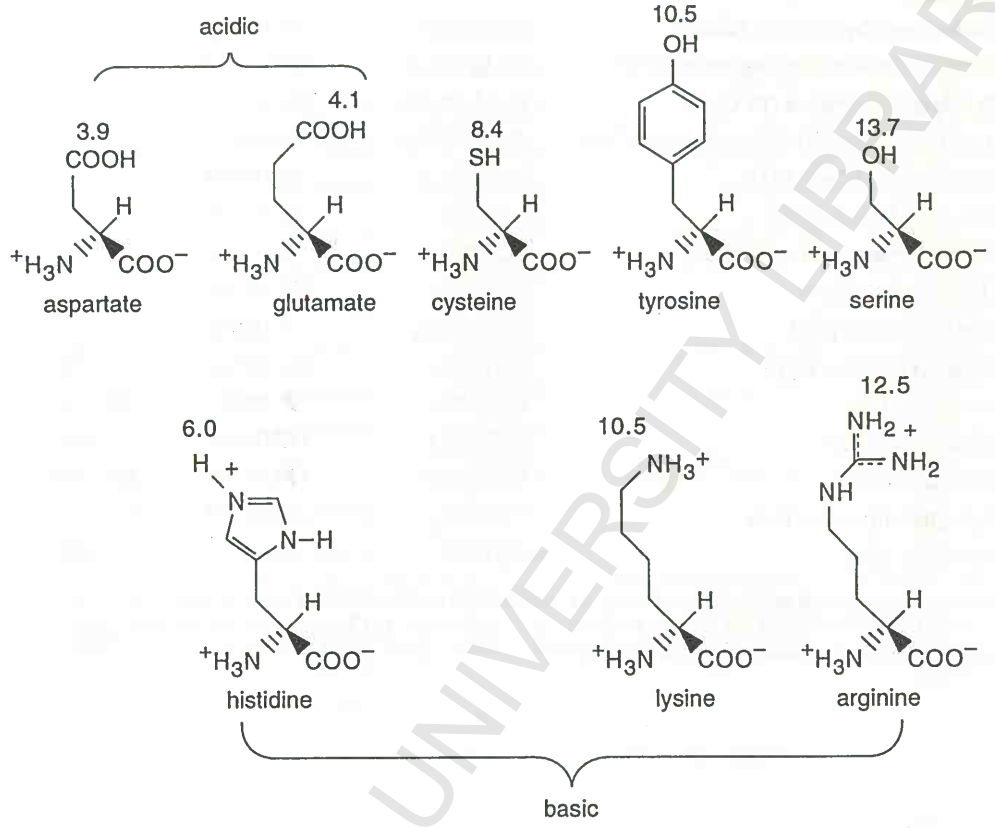
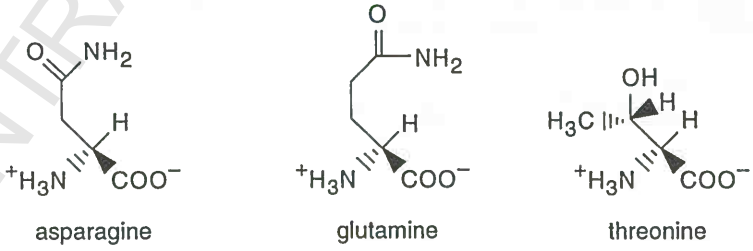
A

Amino acids with dissociable protons



Other amino acids with polar side chains



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B

Nonpolar amino acids

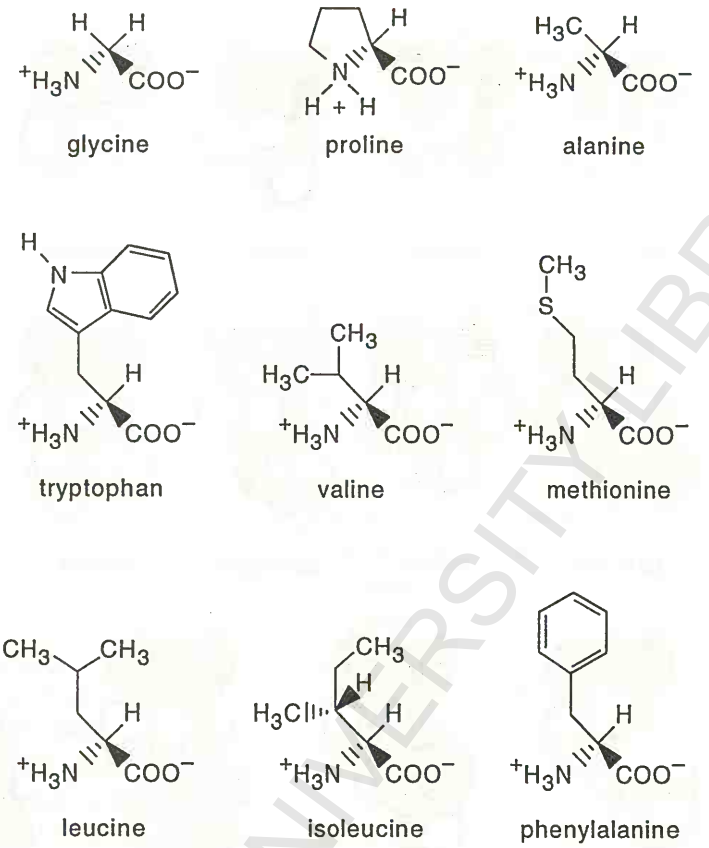

Figure A.1A.1 Line drawings of the amino acids. The amino acids are roughly divided into three groups: amino acids with dissociable protons (**A**), other amino acids with polar side chains (**A**), and nonpolar amino acids (**B**). These groupings are designed to facilitate an understanding of enzymology and the thermodynamics of protein folding.

In this representation, hydrogens are omitted except in showing ionization or stereochemistry. In the case of arginine the delocalized positive charge is indicated by dashed double bonds. At stereocenters, bold lines indicate a group is coming out of the page toward the viewer, while hashed lines indicate that the group goes into the page away from the viewer.

Amino acids with dissociable protons are generally intimately involved in the chemistry of enzymes. Acidic and basic groups can form salt bridges to substrates or to each other. They can also act as proton donors/acceptors in mechanisms that rely on acid/base catalysis. The polar side chains of some of these amino acids (notably cysteine, serine, and histidine) can act as nucleophiles. The pK_a values for the free amino acids are shown, but these values can markedly change when these groups are buried in proteins. The pK_as of the α-amino groups range from 8.7 to 10.7, while the pK_as of the α-carboxylates range from 1.8 to 2.4 (see Table A.1A.1).

Amino acids with polar side chains (**A**) can form hydrogen bonds to substrates or to each other. Cysteine, serine, and tyrosine could also be included in this group, as the ionized forms of these amino acids do not generally perform structural roles in proteins. In general, these amino acids (and the amino acids with dissociable protons) will be found on the surfaces of proteins. Cysteine is an exception, since it is slightly hydrophobic and can often be buried as a disulfide bond.

The nonpolar amino acids (**B**) are often found in the interiors of proteins or in hydrophobic substrate-binding pockets. They interact with one another like jigsaw pieces, forming tight-fitting associations that have a density similar to that of an amino acid crystal. Proline is buried less frequently than might be expected because of its predominance in turns, which are often found on the periphery of a protein.

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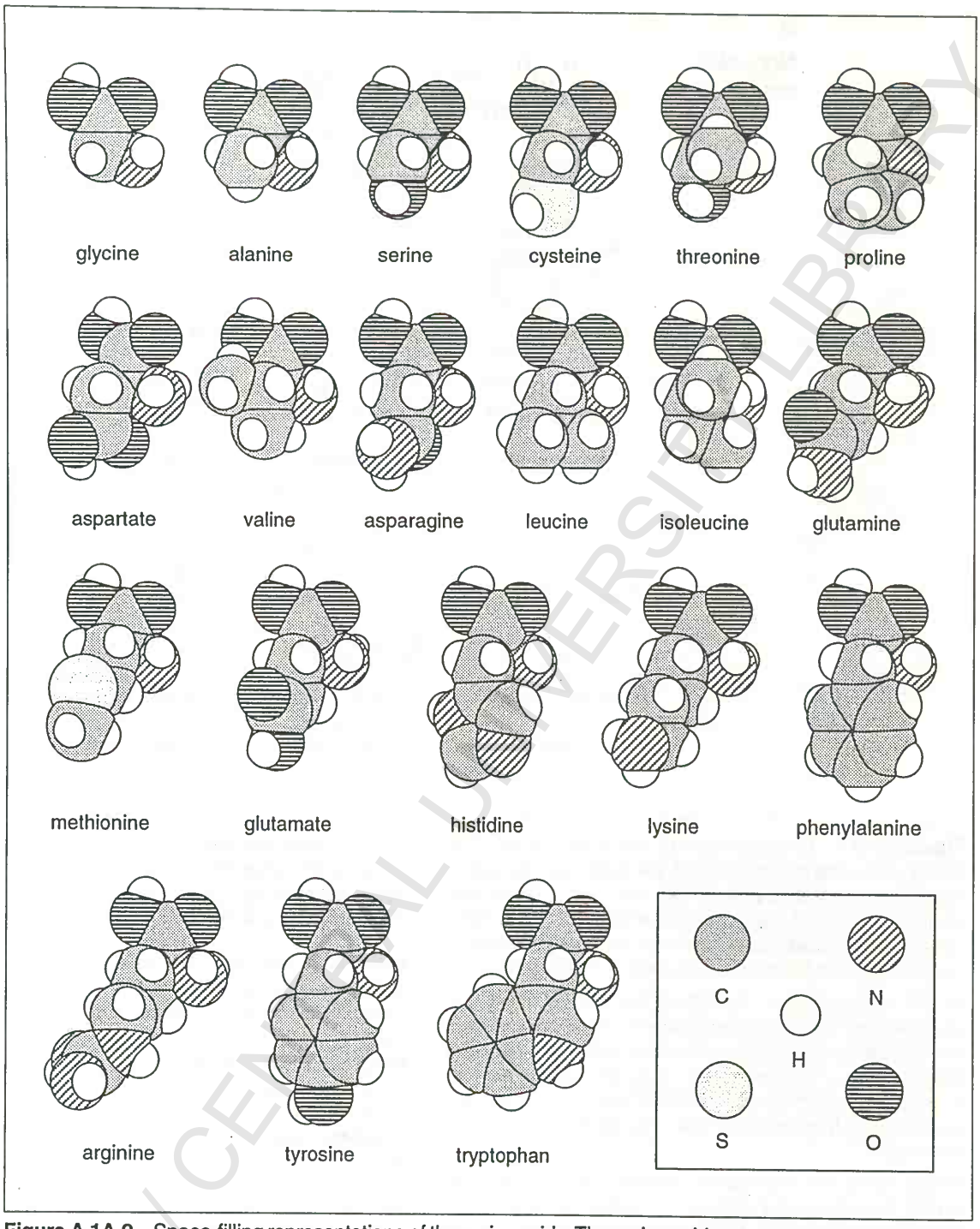

Figure A.1A.2 Space-filling representations of the amino acids. The amino acids are arranged in order of size. The conformations shown maximize the two-dimensional area but are not necessarily the most stable geometries.

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Table A.1A.3 Mass Changes Due to Some Post-Translational Modifications of Peptides and Proteins[a]

Modification[b]	Monoisotopic mass change	Average mass change
Common modifications		
Pyroglutamic acid formation from Gln	−17.0265	−17.0306
Disulfide bond (cystine) formation	−2.0157	−2.0159
C-terminal amide formation from Gly	−0.9840	−0.9847
Deamidation of Asn and Gln	−0.9840	−0.9847
Methylation	14.0157	14.0269
Hydroxylation	15.9949	15.9994
Oxidation of Met	15.9949	15.9994
Proteolysis of a single peptide bond	18.0106	18.0153
Formylation	27.9949	28.0104
Acetylation	42.0106	42.0373
Carboxylation of Asp and Glu	43.9898	44.0098
Phosphorylation	79.9663	79.9799
Sulfation	79.9568	80.0642
Cysteinylation	119.0041	119.1442
Glycosylation with pentoses (Ara, Rib, Xyl)	132.0423	132.1161
Glycosylation with deoxyhexoses (Fuc, Rha)	146.0579	146.1430
Glycosylation with hexosamines (GalN, GlcN)	161.0688	161.1577
Glycosylation with hexoses (Fru, Gal, Glc, Man)	162.0528	162.1424
Modification with lipoic acid (amide bond to lysine)	188.0330	188.3147
Glycosylation with *N*-acetylhexosamines (GalNAc, GlcNAc)	203.0794	203.1950
Farnesylation	204.1878	204.3556
Myristoylation	210.1984	210.3598
Biotinylation (amide bond to lysine)	226.0776	226.2994
Modification with pyridoxal phosphate (Schiff base to lysine)	231.0297	231.1449
Palmitoylation	238.2297	238.4136
Stearoylation	266.2610	266.4674
Geranylgeranlylation	272.2504	272.4741
Glycosylation with *N*-acetylneuraminic acid (sialic acid, NeuAc, NANA, SA)	291.0954	291.2579
Glutathionylation	305.0682	305.3117
Glycosylation with *N*-glycolylneuraminic acid (NeuGe)	307.0903	307.2573
5′-Adenosylation	329.0525	329.2091
Modification with 4′-Phosphopantetheine	339.0780	339.3294
ADP-ribosylation (from NAD)	541.0611	541.3052
Adventitious modifications		
Acrylamide	71.0371	71.0788
Glutathione	304.0712	304.3038
2-Mercaptoethanol	75.9983	76.1192

[a]To obtain the molecular mass of a modified peptide or protein, the appropriate mass changes should be algebraically added to the molecular mass calculated for the unmodified molecule.

[b]A more extensive list of modifications is available from the Delta mass site at http://www.medstv.unimelb.edu.au/WWWDOCS/SVIMRDocs/MassSpec/deltamassV2.html.

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Table A.1A.4 Mass Increments for Common Carbohydrate Structures

Sugars[b]	In-chain mass[a]					
	Monoisotopic			Chemical average		
	1^+	2^+	3^+	1^+	2^+	3^+
Deoxyhexose	146.058	73.0	48.7	146.144	73.0	48.7
Hexose	162.053	81.0	54.0	162.143	81.1	54.0
Hexosamine	161.069	80.5	53.7	161.158	80.6	53.7
N-acetylhexosamine	203.080	101.6	67.7	203.196	101.6	67.7
N-acetylneuraminic acid	291.095	145.6	97.0	291.259	145.6	97.1
N-glycolylneuraminic acid	307.090	153.6	102.4	307.257	153.6	102.4
Hex-HexNAc	365.132	182.6	121.7	365.339	182.7	121.8
NeuAc-Hex-HexNAc	656.228	328.1	218.7	656.598	328.3	218.9
$(Hex)_3$-HexNAc-HexNAc	892.317	446.2	297.4	892.821	446.4	297.6
$(Hex)_3$-HexNAc-(dHex)HexNAc	1038.375	519.2	346.1	1038.965	519.5	346.3
Biantennary (1 dHex; 0 NeuAc)	1768.640	884.3	589.5	1769.643	884.8	589.9
Triantennary (1 dHex; 0 NeuAc)	2133.772	1066.9	711.3	2134.982	1067.5	711.7
Tetraantennary (1 dHex; 0 NeuAc)	2498.904	1249.5	833.0	2500.321	1250.2	833.4

[a]End-group mass (+18.011 for monoisotopic, +18.015 for chemical average) + in-chain mass = molecular mass of carbohydrate. Determined M_r of peptide (attachment site = Asn) – in-chain mass of carbohydrate.

[b]Abbreviations: dHex, deoxyhexose; Hex, hexose; HexN, hexosamine; HexNAc, *N*-acetylhexosamine; NeuAc, *N*-acetylneuraminic acid.

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Commonly Used Detergents

Detergents are polar lipids that are soluble in water. The presence of both a hydrophobic and hydrophilic portion makes these compounds very useful for lysis of lipid membranes, solubilization of antigens, and washing of immune complexes.

TYPES OF DETERGENTS

A large variety of detergents are available (Helenius et al., 1979). For biochemical studies, they are usually categorized according to the type of hydrophilic group they contain—anionic, cationic, amphoteric, or nonionic. Tables A.1B.1 and A.1B.2 list commonly used members of each type. In general, nonionic and amphoteric detergents are less denaturing for proteins than ionic detergents. Sodium cholate and sodium deoxycholate are the least denaturing of the commonly used ionic detergents.

Two properties of detergents are important in their consideration for biological studies: the critical micelle concentration (CMC) and the micelle molecular weight (Table A.1B.1). The CMC is the concentration at which monomers of detergent molecules combine to form micelles; each detergent micelle has a characteristic micelle molecular weight. Detergents with a high micelle molecular weight, such as nonionic detergents, are difficult to remove from samples by dialysis. The CMC and the micelle molecular weight will vary depending on the buffer, salt concentration, pH, and temperature. In general, adding salt will lower the CMC and raise the micelle size.

Table A.1B.1 Physical Properties of Commonly Used Detergents[a,b]

Detergent	mp (°C)	Molecular weight (Da)		CMC	
		Monomer	Micelle	% (w/v)	M
Anionic					
SDS	206	288	18,000	0.23	8.0×10^{-3}
Cholate	201	430	4,300	0.60	1.4×10^{-2}
Deoxycholate	175	432	4,200	0.21	5.0×10^{-3}
Cationic					
C_{16}TAB	230	365	62,000	0.04	1.0×10^{-3}
Amphoteric					
LysoPC	—	495	92,000	0.0004	7.0×10^{-6}
CHAPS	157	615	6,150	0.49	1.4×10^{-3}
Zwittergent 3-14	—	364	30,000	0.011	3.0×10^{-4}
Nonionic					
Octyl glucoside	105	292	8,000	0.73	2.3×10^{-2}
Digitonin	235	1,229	70,000	—	—
$C_{12}E_8$	—	542	65,000	0.005	8.7×10^{-5}
Lubrol PX	—	582	64,000	0.006	1.0×10^{-4}
Triton X-100	—	650	90,000	0.021	3.0×10^{-4}
Nonidet P-40	—	603	90,000	0.017	3.0×10^{-4}
Tween 80	—	1,310	76,000	0.002	1.2×10^{-5}

[a] Reprinted with permission from IRL Press (see Jones et al., 1987).

[b] Abbreviations: C_{16}TAB, hexadecyl trimethylammonium bromide; CMC, critical micelle concentration; LysoPC, lysophosphatidylcholine; mp, melting point; SDS, sodium dodecyl sulfate.

Contributed by John E. Coligan

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Table A.1B.2 Chemical Properties of Commonly Used Detergents[a,b]

Property	Ionic detergents							Nonionic detergents						
	SDS	CHO	DOC	C_{16}	LYS	CHA	ZWI	OGL	DIG	C_{12}	LUB	TNX	NP-40	T80
Strongly denaturing[c]	+	–	–	+	+/–	–	+/–	–	–	–	–	–	–	–
Dialyzable	+	+	+	+	–	+	+/–	+	–	–	–	–	–	–
Ion exchangeable[d]	+	+	+	+	–	–	–	–	–	–	–	–	–	–
Complexes ions	+	+	+	–	–	–	–	–	–	+/–	+/–	+/–	+/–	+/–
Strong A_{280}	–	–	–	–	–	–	–	–	–	–	–	+	+	–
Assay interference	–	–	–	–	–	–	–	–	–	–	+/–	+/–	+/–	+/–
Cold precipitates	+	–	+	+	–	–	–	–	–	–	–	–	–	–
High cost	–	–	–	–	+	+	+	+	+	+	–	–	–	–
Availability	+	+	+	+	+	+	+/–	+	+	+/–	+	+	+	+
Toxicity	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ease of purification	+	+	+	+	+/–	+	+	–	+	–	–	–	–	–
Radiolabeled	+	+	+	–	+	–	–	+	–	+	+	+	+	+
Defined composition	+	+	+	+	+	+	+	+		–	–	–	–	–
Auto-oxidation	–	–	–	–	–	–	–	–		+	+	+	+	+

[a]Adapted from IRL Press (see Jones et al., 1987).

[b]Abbreviations: C_{12}, $C_{12}E_8$; C_{16}, hexadecyl trimethylammonium bromide; CHA, CHAPS; CHO, cholate; DIG, digitonin; DOC, deoxycholate; LUB, lubrol PX; LYS, lysophosphatidylcholine; NP-40, Nonidet P-40; OGL, octyl glucoside; SDS, sodium dodecyl sulfate; T80, Tween 80; TNX, Triton X-100; ZWI, Zwittergent 3-14.

[c] Denaturing refers to disruption of secondary and tertiary protein structure.

[d]Ionic detergents are unsuitable for ion-exchange chromatography (*UNIT 8.2*).

CHOICE OF DETERGENTS

Ionic detergents are very good solubilizing agents, but they tend to denature proteins by destroying native three-dimensional structures. This denaturing ability is useful for SDS-PAGE (*UNIT 10.1*), but is detrimental where native structure is important, as when functional activities must be retained (antibody activity is usually retained in <0.1% SDS). Nonionic and mildly ionic detergents are less denaturing and can often be used to solubilize membrane proteins while retaining protein-protein interactions. The following detergent properties are detrimental in certain procedures:

1. Phenol-containing detergents (e.g., Triton X-100 and NP-40) have a high absorbance at 280 nm and hence interfere with protein monitoring during chromatography (most ionic detergents do not absorb at 280 nm; Brij- and Lubrol-series detergents are nonionic detergents that do not have substantial absorbance at 280 nm). Phenol-containing detergents also induce precipitation in the Folin protein assay (but they can be used with the Bradford protein assay; *UNIT 3.4*). Finally, they are readily iodinated and so should not be present during radioiodination.

2. Many detergents have a very high micelle molecular weight (Table A.1B.1), which makes their use in gel filtration impossible since protein sizes are insignificant relative to the micelle size. In addition, such detergents cannot be readily removed by dialysis.

3. Sodium cholate and sodium deoxycholate are insoluble below pH 7.5 or above an ionic strength of 0.1%. SDS will often crystallize below 20°C.

4. Ionic detergents interfere with nondenaturing electrophoresis (*UNIT 10.3*) and isoelectric focusing (*UNIT 10.2*).

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Detergents can be removed or exchanged for other detergents by a variety of procedures (Hjelmeland, 1979; Furth et al., 1984; Harlow and Lane, 1988). Ionic and amphoteric detergents can usually be removed by dialysis *UNIT 4.4*. Pierce makes Extracti-Gel D for removing a variety of detergents from protein solutions (*Pierce Immunotechnology Catalog* and *Handbook on Protein Modification*).

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Contributed by John E. Coligan
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Conversion Factors and Half-Life Information for Radioactivity

Discussion of radioactivity may use different terms for measurements of radioactivity and dose. Conversion factors for these terms are presented in Table A.1C.1. In addition, when investigators are working with a particular radiolabeled compound, it is necessary to calculate the amount of remaining radioactivity based on the amount of time that has elapsed from the reference date and the half life of the radioisotope (see Fig. A.1C.1). Decay factors for the commonly used shorter half life radioisotopes are presented in Table A.1C.2. For information about working with radioisotopes, see Guide for Safe Handling of Radioisotopes (*APPENDIX 2B*).

Table A.1C.1 Conversion Factors for Radioactivity

Measurement of Radioactivity

The SI unit for measurement of radioactivity is the Becquerel:

1 Becquerel (Bq) = 1 disintegration per second

The more commonly encountered unit is the Curie (Ci):

$1\ Ci = 3.7 \times 10^{10}\ Bq$

$= 2.22 \times 10^{12}$ disintegrations per minute (dpm)

1 millicurie (mCi) = 3.7×10^{7} Bq = 2.22×10^{9} dpm

1 microcurie (μCi) = 3.7×10^{4} Bq = 2.22×10^{6} dpm

Conversion factors:

1 day = 1.44×10^{3} min = 8.64×10^{4} sec

1 year = 5.26×10^{5} min = 3.16×10^{7} sec

counts per minute (cpm) = dpm × (counting efficiency)

Measurement of Dose

The SI unit for energy absorbed from radiation is the Gray (Gy):

1 Gy = 1 joule/kg

Older units of absorbed energy are the rad and Roentgen (R):

1 rad = 100 ergs/g = 10^{-2} Gy

1 R = 0.877 r in air = 0.93 – 0.98 r in water and tissue

The SI unit for radiation dosage is the Sievert (Sv), which takes into account the empirically determined relative biological effectiveness (RBE) of a given form of radiation:

dosage [Sv] = RBE × dosage [Gy]

$$RBE = \frac{\text{(biological effect of a dose of standard radiation [Gy])}}{\text{(biological effect of a dose of other radiation [Gy])}}$$

RBE = 1 for commonly encountered radionuclides

The older unit for dosage is the rem (Roentgen-equivalent-man):

1 rem = 0.01 Sv

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Table A.1C.2 Decay factors for calculating the amount of radioactivity present at a given time after a reference date. For example, a vial containing 1.85 MBq (50 μCi) of an ^{35}S-labeled compound on the reference date will have the following activity 33 days later: $1.85 \times 0.770 = 1.42$ MBq; $50 \times 0.770 = 38.5$ μCi.

^{125}I Half-life: 60.0 days

Days	0	2	4	6	8	10	12	14	16	18
0	1.000	0.977	0.955	0.933	0.912	0.891	0.871	0.851	0.831	0.812
20	0.794	0.776	0.758	0.741	0.724	0.707	0.691	0.675	0.660	0.645
40	0.630	0.616	0.602	0.588	0.574	0.561	0.548	0.536	0.524	0.512
60	0.500	0.489	0.477	0.467	0.456	0.445	0.435	0.425	0.416	0.406
80	0.397	0.388	0.379	0.370	0.362	0.354	0.345	0.338	0.330	0.322
100	0.315	0.308	0.301	0.294	0.287	0.281	0.274	0.268	0.262	0.256
120	0.250	0.244	0.239	0.233	0.228	0.223	0.218	0.213	0.208	0.203
140	0.198	0.194	0.189	0.185	0.181	0.177	0.173	0.169	0.165	0.161
160	0.157	0.154	0.150	0.147	0.144	0.140	0.137	0.134	0.131	0.128
180	0.125	0.122	0.119	0.117	0.114	0.111	0.109	0.106	0.104	0.102
200	0.099	0.097	0.095	0.093	0.090	0.088	0.086	0.084	0.082	0.081
220	0.079	0.077	0.075	0.073	0.072	0.070	0.069	0.067	0.065	0.064
240	0.063	0.061	0.060	0.058	0.057	0.056	0.054	0.053	0.052	0.051

^{32}P Half-life: 14.3 days

Days	Hours 0	12	24	36	48	60	72	84
0	1.000	0.976	0.953	0.930	0.908	0.886	0.865	0.844
4	0.824	0.804	0.785	0.766	0.748	0.730	0.712	0.695
8	0.679	0.662	0.646	0.631	0.616	0.601	0.587	0.573
12	0.559	0.546	0.533	0.520	0.507	0.495	0.483	0.472
16	0.460	0.449	0.439	0.428	0.418	0.408	0.398	0.389
20	0.379	0.370	0.361	0.353	0.344	0.336	0.328	0.320
24	0.312	0.305	0.298	0.291	0.284	0.277	0.270	0.264
28	0.257	0.251	0.245	0.239	0.234	0.228	0.223	0.217
32	0.212	0.207	0.202	0.197	0.192	0.188	0.183	0.179
36	0.175	0.170	0.166	0.162	0.159	0.155	0.151	0.147
40	0.144	0.140	0.137	0.134	0.131	0.127	0.124	0.121
44	0.119	0.116	0.113	0.110	0.108	0.105	0.102	0.100
48	0.098	0.095	0.093	0.091	0.089	0.086	0.084	0.082
52	0.080	0.078	0.077	0.075	0.073	0.071	0.070	0.068

^{131}I Half-life: 8.04 days

Days	Hours 0	6	12	18	24	30	36	42	48	54	60	66
0	1.000	0.979	0.958	0.937	0.917	0.898	0.879	0.860	0.842	0.824	0.806	0.789
3	0.772	0.756	0.740	0.724	0.708	0.693	0.678	0.664	0.650	0.636	0.622	0.609
6	0.596	0.583	0.571	0.559	0.547	0.533	0.524	0.513	0.502	0.491	0.481	0.470
9	0.460	0.450	0.441	0.431	0.422	0.413	0.405	0.396	0.387	0.379	0.371	0.363
12	0.355	0.348	0.340	0.333	0.326	0.319	0.312	0.306	0.299	0.293	0.286	0.280
15	0.274	0.269	0.263	0.257	0.252	0.246	0.241	0.236	0.231	0.226	0.221	0.216
18	0.212	0.207	0.203	0.199	0.194	0.190	0.186	0.182	0.178	0.175	0.171	0.167
21	0.164	0.160	0.157	0.153	0.150	0.147	0.144	0.141	0.138	0.135	0.132	0.129
24	0.126	0.124	0.121	0.118	0.116	0.113	0.111	0.109	0.106	0.104	0.102	0.100
27	0.098	0.095	0.093	0.091	0.089	0.088	0.086	0.084	0.082	0.080	0.079	0.077
30	0.075	0.074	0.072	0.071	0.069	0.068	0.066	0.065	0.064	0.063	0.061	0.059
33	0.058	0.057	0.056	0.054	0.053	0.052	0.051	0.050	0.049	0.048	0.047	0.046
36	0.045	0.044	0.043	0.042	0.041	0.040	0.039	0.039	0.038	0.037	0.036	0.035

^{35}S Half-life: 87.4 days

Weeks	Days 0	1	2	3	4	5	6
0	1.000	0.992	0.984	0.976	0.969	0.961	0.954
1	0.946	0.939	0.931	0.924	0.916	0.909	0.902
2	0.895	0.888	0.881	0.874	0.867	0.860	0.853
3	0.847	0.840	0.833	0.827	0.820	0.814	0.807
4	0.801	0.795	0.788	0.782	0.776	0.770	0.764
5	0.758	0.752	0.746	0.740	0.734	0.728	0.722
6	0.717	0.711	0.705	0.700	0.694	0.689	0.683
7	0.678	0.673	0.667	0.662	0.657	0.652	0.646
8	0.641	0.636	0.631	0.626	0.621	0.616	0.612
9	0.607	0.602	0.597	0.592	0.588	0.583	0.579
10	0.574	0.569	0.565	0.560	0.556	0.552	0.547
11	0.543	0.539	0.534	0.530	0.526	0.522	0.518
12	0.514	0.510	0.506	0.502	0.498	0.494	0.490

^{33}P Half-life: 25.4 days

Days	0	1	2	3	4	5	6	7	8	9
0	1.000	0.973	0.947	0.921	0.897	0.872	0.849	0.826	0.804	0.782
10	0.761	0.741	0.721	0.701	0.683	0.664	0.646	0.629	0.612	0.595
20	0.579	0.564	0.549	0.534	0.520	0.506	0.492	0.479	0.466	0.453
30	0.441	0.429	0.418	0.406	0.395	0.385	0.374	0.364	0.355	0.345
40	0.336	0.327	0.318	0.309	0.301	0.293	0.285	0.277	0.270	0.263
50	0.256	0.249	0.242	0.236	0.229	0.223	0.217	0.211	0.205	0.200
60	0.195	0.189	0.184	0.179	0.174	0.170	0.165	0.161	0.156	0.152
70	0.148	0.144	0.140	0.136	0.133	0.129	0.126	0.122	0.119	0.116
80	0.113	0.110	0.107	0.104	0.101	0.098	0.096	0.093	0.091	0.088
90	0.086	0.084	0.081	0.079	0.077	0.075	0.073	0.071	0.069	0.067
100	0.065	0.064	0.062	0.060	0.059	0.057	0.055	0.054	0.053	0.051
110	0.050	0.048	0.047	0.046	0.045	0.043	0.042	0.041	0.040	0.039
120	0.038	0.037	0.036	0.035	0.034	0.033	0.032	0.031	0.030	0.030

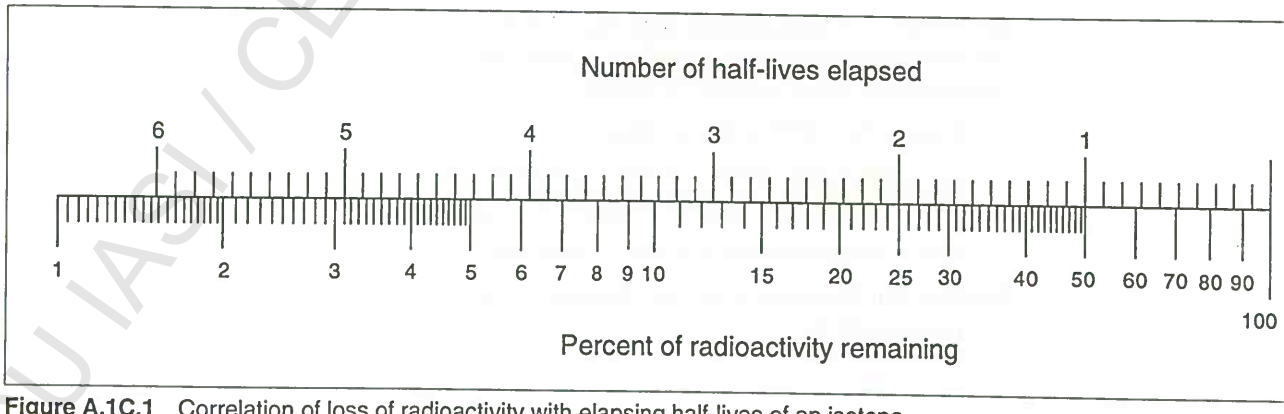

Figure A.1C.1 Correlation of loss of radioactivity with elapsing half-lives of an isotope.

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LABORATORY GUIDELINES, EQUIPMENT, AND STOCK SOLUTIONS

Laboratory Safety

Persons carrying out the protocols in this manual may encounter the following hazardous or potentially hazardous materials (1) pathogenic and infectious biological agents and (2) toxic chemicals and carcinogenic, mutagenic, or teratogenic reagents (Table A.2A.1). Most governments regulate the use of these materials; it is essential that they be used in strict accordance with local and national regulations. Cautionary notes are included in many instances throughout the manual, and some specific guidelines are provided below (and in references therein). However, we emphasize that users must proceed with the prudence and precautions associated with good laboratory practice, under the supervision of personnel responsible for implementing laboratory safety programs at their institutions. Guidelines for the safe use of radioisotopes are presented in *APPENDIX 2B*.

SAFE USE OF BIOHAZARDS AND INFECTIOUS BIOLOGICAL AGENTS

Precautions described in this section should be applied to the routine handling of viable pathogenic microorganisms, as well as all human-derived materials, because they may harbor dangerous pathogens such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and a host of bacterial pathogens. In addition to the guidelines provided herein, experimenters can find a wealth of information about handling infectious agents in the government publications *Biosafety in Microbiological and Biomedical Laboratories* and *Working Safely with HIV in the Research Laboratory* (see Literature Cited).

Routine Precautions When Working with Biohazards

The following practices are recommended for all laboratories handling potentially dangerous microorganisms, whether pathogenic or not:

1. Decontaminate all work surfaces after each working day using an appropriate disinfectant. Decontaminate all spills of viable material. See discussion under Disinfectants for Biohazards.
2. Decontaminate all liquid or solid wastes that have come in contact with viable material.
3. Do not pipet by mouth.
4. Do not allow eating, drinking, smoking, or application of cosmetics in the work area. Do not store food in refrigerators that contain laboratory supplies.
5. Wash hands with disinfectant soap or detergent after handling viable materials and before leaving the lab. Do not handle telephones, doorknobs, or other common utensils without disinfecting hands.
6. When handling viable materials, minimize creation of aerosols.
7. Wear lab coats (preferably disposable) when in work area, but do not wear them away from the work area.
8. Wear disposable latex gloves when handling viable materials. These should be disposed of as biohazardous waste. Change gloves if they are directly contaminated.

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9. Control pest populations. Windows in the lab that can be opened must be equipped with screens to exclude insects.
10. Use furniture that is easy to clean—i.e., with smooth, waterproof surfaces and as few seams as possible.
11. Keep biohazard waste in covered containers free from leaks. Use orange bags or red bags as required by institutional procedure (see discussion under Disposal of Biohazards). Autoclave and dump hazardous waste without undue delay.

Disinfectants for Biohazards

Major laboratory suppliers sell disinfectants based on quaternary ammonium compounds that are acceptable for routine biohazard decontamination (see *SUPPLIERS APPENDIX*). These include Roccal (Baxter), Vesphene II (Fisher), and industrial disinfectants such as concentrated Lysol. 10% chlorine bleach may also be used for decontamination. An antimicrobial liquid soap (e.g., Vionex; Fisher) should be provided in a dispenser near the sink so that no one need handle the outside of the container to use it.

Disposal of Biohazards

Most institutions have defined procedures for disposal of biohazardous waste, but the following are common to all of these systems:

1. All contaminated material should be placed in autoclavable bags, which should be contained in a plastic trash pail or wire frame. If large numbers of disposable pipets or other pointed instruments are being used, it may be necessary to double-bag the material. Autoclavable biohazard bags are sold by all major laboratory supply houses. In some institutions it is necessary to color code the biohazard waste (e.g., orange bags for less dangerous waste and red ones for suspected HIV-containing material). All of these bags are marked with the universal biohazard symbol.
2. At time of disposal, the bags are loosely closed (not completely sealed) with temperature-sensitive autoclave tape (also widely available from supply houses), placed in an autoclavable basin, and sterilized at 121°C. When the tape indicates that sterilization temperature has been achieved, it is then possible to dispose of the waste by ordinary means.
3. At many institutions, contaminated hypodermic needles, scalpels, broken glass, and other sharp objects must be disposed of separately. These must be placed in appropriate "sharps" containers (e.g., Baxter), which may be autoclaved when full.

SAFE USE OF HAZARDOUS CHEMICALS

It is not possible in the space available to list all the precautions required for handling hazardous chemicals. Many texts have been written about laboratory safety (see Literature Cited and Key References). Obviously, all national and local laws should be obeyed, as well as all institutional regulations. Controlled substances are regulated by the Drug Enforcement Administration. By law, Material Safety Data Sheets must be readily available. All laboratories should have a Chemical Hygiene Plan (29CFR Part 1910.1450); institutional safety officers should be consulted as to its implementation. Help is (or should be) available from your institutional Safety Office. Use it.

Chemicals should be stored properly in compliance with regulations established by local fire departments for storage of chemicals. For example, flammable chemicals (e.g., ethanol, methanol, acetone, methyl ethyl ketone, petroleum distillates, toluene, benzene, and other materials labeled flammable) should be stored in approved flammable storage

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cabinets; flammable chemicals requiring refrigeration should be stored in explosion-proof refrigerators. Oxidizers should be segregated from other chemicals, and corrosive acids (e.g., sulfuric, hydrochloric, nitric, perchloric, and hydrofluoric acids) should also be stored in a separate cabinet, well-removed from the flammable organics.

Facilities should be appropriate for the handling of hazardous chemicals. In particular, hazardous chemicals should be handled only in chemical fume hoods, not in laminar flow cabinets. The functioning of the fume hoods should be checked periodically. Laboratories should also be equipped with safety showers and eye-wash facilities. Again, this equipment should be tested periodically to ensure that it functions correctly. Other safety equipment may be required depending on the nature of the materials being handled. In addition, researchers should be trained in the proper procedures for handling hazardous chemicals as well as other laboratory operations: e.g., handling of compressed gases, use of cryogenic liquids, operation of high-voltage power supplies, and operation of autoclaves.

Before starting work, a plan for dealing with spills or accidents should be in place; coming up with a good plan on the spur of the moment is very difficult. For example, have appropriate decontaminating or neutralizing agents prepared and close at hand (e.g., keep a bucket containing >1 kg of sodium or calcium carbonate handy to neutralize spills of corrosive acids). Small spills can probably be cleaned up by the researcher. In the case of larger spills, the area should be evacuated and help should be sought from those experienced and equipped for dealing with spills, e.g., the institutional Safety Office.

Protective equipment should include, at a minimum, eye protection, a lab coat, and gloves. In certain circumstances other items of protective equipment may be necessary (e.g., a face shield). Different types of gloves exhibit different chemical resistance properties; a listing of the properties of gloves is available (Forsberg et al., 1989). Gloves should, however, be regarded as the last line of defense and should be changed if they become contaminated, because many types of chemicals pass relatively freely through rubber. If possible, handling procedures should be designed so the gloves do not become contaminated. All common-sense precautions should be observed: e.g., do not pipet by mouth, keep unauthorized persons away from hazardous chemicals, do not eat or drink in the lab, wear proper clothing in the lab—sandals, open-toed shoes, and shorts are not appropriate.

Order hazardous chemicals only in quantities that are likely to used in a reasonable time. Buying large quantities at a lower unit cost is no bargain if someone (perhaps you) has to pay to dispose of surplus quantities. Substitute alcohol-filled thermometers for mercury-filled thermometers, which are a hazardous chemical spill waiting to happen.

Although any number of chemicals commonly used in laboratories are toxic if used improperly, there are a number of reagents whose toxic properties require special mention. Chemicals that exhibit carcinogenic, corrosive, flammable, lachrymatory, mutagenic, oxidizing, teratogenic, toxic, or other hazardous properties are listed in Table A.2A.1. Chemicals listed as carcinogenic range from those accepted by expert review groups as causing cancer in humans to those for which only minimal evidence of carcinogenicity exists. No effort has been made to differentiate the carcinogenic potential of the compounds in Table A.2A.1. Oxidizers may react violently with oxidizable material, e.g., hydrocarbons, wood, and cellulose. Before using any of these chemicals, thoroughly investigate all of its characteristics. Material Safety Data Sheets are readily available; they list some hazards but vary widely in quality. A number of texts describing hazardous properties are listed in Literature Cited. In particular *Sax's Dangerous Properties of Industrial Materials*, 8th ed. (Lewis, 1992) and the *Handbook of Reactive Chemical Hazards*, 5th ed. (Urben, 1995) give comprehensive listings of known hazardous properties. However, these texts list only the known properties. Many chemicals have been tested

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Table A.2A.1 Hazardous Chemicals Commonly Used in Laboratories[a]

Chemical	Hazards[b]	Remarks[c]
Acetic acid, glacial	Corrosive, flammable liquid	
Acetonitrile	Flammable liquid, teratogenic, toxic	
Acridine orange	Carcinogenic, mutagenic	
Acrylamide	Carcinogenic, toxic	Use dust mask; polyacrylamide gels contain residual acrylamide monomer and should be handled with gloves; acrylamide may polymerize with violence on melting at 86°C
Ammoniacal silver solution	Potentially explosive	Prepare fresh solution daily; discard promptly with water or in accordance with local regulations
Ammonium hydroxide, concentrated	Corrosive, lachrymatory, toxic	
Benzidine (BDB)	Carcinogenic, toxic	See Basic Protocol 1
Bisacrylamide	Toxic	
Brilliant blue R	Carcinogenic, mutagenic	
Cetylpyridinium chloride (CPC)	Toxic	
Cetyltrimethylammonium bromide (CTAB)	Corrosive, teratogenic, toxic	
Chloroform	Carcinogenic, teratogenic, toxic	
Chlorotrimethylsilane	Carcinogenic, corrosive, flammable liquid, toxic	Reacts violently with water; see Basic Protocol 2
Chromic/sulfuric acid cleaning solution	Carcinogenic, corrosive, oxidizer, toxic	
Congo red	Mutagenic, teratogenic	
Coomassie brilliant blue G	Mutagenic	
Cresyl violet acetate	Mutagenic	
Cyanides (e.g., KCN, NaCN)	Toxic	Contact with acid will liberate HCN gas; see Basic Protocol 3
Cyanogen bromide (CNBr)	Toxic	See Basic Protocol 3
2′-Deoxycoformycin (dCF, pentostatin)	Teratogenic, toxic	
Diaminobenzidine (DAB)	Carcinogenic	See Basic Protocol 1
1,4-Diazabicyclo[2,2,2]-octane (DABCO)	Toxic	Forms an explosive complex with hydrogen peroxide
Dichloroacetic acid (DCA)	Carcinogenic, corrosive, toxic	
Dichloromethane	Carcinogenic, mutagenic, teratogenic, toxic	
Diethylamine (DEA)	Corrosive, flammable liquid, toxic	
Diethylpyrocarbonate (DEPC)	Carcinogenic, toxic	

continued

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Table A.2A.1 Hazardous Chemicals Commonly Used in Laboratories[a], continued

Chemical	Hazards[b]	Remarks[c]
Diethyl sulfate	Carcinogenic, teratogenic, toxic	
Diisopropyl fluorophosphate (DFP)	Neurotoxic, volatile, mutagenic	Can be diluted in aqueous solutions up to ~10 mM; inactivate by adding 1 N NaOH so that pH is ≥12; see Basic Protocol 4
Dimethyldichlorosilane	Corrosive, flammable liquid, toxic	See Basic Protocol 2
Dimethyl sulfate (DMS)	Carcinogenic, toxic	
Diphenylamine (DPA)	Teratogenic, toxic	
2,5-Diphenyloxazole (PPO)	Toxic	
Dithiothreitol (DTT)	Toxic	
Erythrosin B	Carcinogenic, mutagenic	
Ether	Flammable liquid, toxic	May form explosive peroxides on standing; do not dry with NaOH or KOH
Ethidium bromide (EtBr)	Mutagenic, toxic	See Basic Protocol 5
Ethyl methanesulfonate (EMS)	Carcinogenic, toxic	
Fluoroorotic acid (FOA)	Toxic	
Formaldehyde	Carcinogenic, flammable liquid, teratogenic, toxic	
Formamide	Teratogenic, toxic	
Formic acid	Corrosive, toxic	May explode when heated to >180°C in a sealed tube
Glutaraldehyde	Corrosive, teratogenic, toxic	
Guanidinium thiocyanate	Toxic	
Hydrazine	Carcinogenic, corrosive, toxic	May explode on distillation or react violently with traces of metal compounds or oxidizers; see Basic Protocol 6
Hydrochloric acid, concentrated	Corrosive, teratogenic, toxic	
Hydrogen peroxide (30%)	Carcinogenic, corrosive, mutagenic, oxidizer	Should not be brought into contact with organic materials, which may form explosive peroxides; may decompose violently in contact with metals, salts, or oxidizable materials; see Basic Protocol 7
Hydroxylamine	Corrosive, flammable, mutagenic, toxic	Explodes in air at >70°C
3-β-Indoleacrylic acid (IAA)	Carcinogenic	
Iodine	Corrosive, toxic	See Basic Protocol 8
Iodoacetamide	Carcinogenic, mutagenic, toxic	
Lead compounds	Carcinogenic, toxic	
2-Mercaptoethanol (2-ME)	Stench, toxic	
Mercury compounds	Teratogenic, toxic	See Basic Protocol 9

continued

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Table A.2A.1 Hazardous Chemicals Commonly Used in Laboratories[a], continued

Chemical	Hazards[b]	Remarks[c]
Methionine sulfoximine (MSX)	Teratogenic, toxic	
Methotrexate (amethopterin)	Carcinogenic, mutagenic, teratogenic, toxic	
Methylene blue	Mutagenic, toxic	
Methyl methanesulfonate (MMS)	Carcinogenic, toxic	
Mycophenolic acid (MPA)	Teratogenic, toxic	
Neutral red	Mutagenic	
Nitric acid, concentrated	Corrosive, oxidizer, teratogenic, toxic	
Nitroblue tetrazolium (NBT)	Toxic	
Paraformaldehyde	Toxic	
Phenol	Carcinogenic, corrosive, teratogenic, toxic	Readily absorbed through the skin
Phenylmethylsulfonyl fluoride (PMSF)	Corrosive, toxic	Degrade by adding 1 M NaOH stats pH is ≥12; see Basic Protocol 10
Piperidine	Flammable liquid, teratogenic, toxic	
Potassium hydroxide, concentrated	Corrosive, toxic	Adding the solid to water produces a highly exothermic reaction
Propane sultone	Carcinogenic, toxic	
Propidium iodide (PI)	Mutagenic	See Basic Protocol 5
Pyridine	Flammable liquid, toxic	
Seleno-L-methionine	Highly toxic	
Sodium azide	Carcinogenic, toxic	Adding acid liberates explosive volatile, toxic hydrazoic acid; can form explosive heavy metal azides, e.g., with plumbing fixtures—*do not* discharge down drain; see Basic Protocol 11
Sodium deoxycholate (Na-DOC)	Carcinogenic, teratogenic, toxic	
Sodium hydroxide, concentrated	Corrosive, toxic	Adding the solid to water produces highly exothermic reaction
Sodium nitrite	Carcinogenic	
Sulfuric acid, concentrated	Corrosive, oxidizer, teratogenic, toxic	Dissolving concentrated sulfuric acid in water produces a highly exothermic reaction—always add concentrated sulfuric acid to water, never water to the acid
Tetramethylammonium chloride (TMAC)	Toxic	
N,N,N′,N′-Tetramethyl-ethylenediamine (TEMED)	Corrosive, flammable liquid, toxic	
Toluene	Flammable liquid, teratogenic, toxic	
Trichloroacetic acid (TCA)	Carcinogenic, corrosive, teratogenic, toxic	

continued

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Table A.2A.1 Hazardous Chemicals Commonly Used in Laboratories[a], continued

Chemical	Hazards[b]	Remarks[c]
Triethanolamine acetate (TEA)	Carcinogenic, toxic	
Trifluoroacetic acid (TFA)	Corrosive, toxic	
Trimethyl phosphate (TMP)	Carcinogenic, mutagenic, teratogenic	May explode on distillation
Trypan blue	Carcinogenic, mutagenic, teratogenic	
Xylenes	Flammable liquid, teratogenic, toxic	

[a]For extensive information on the hazards of these and other chemicals as well as cautionary details, see Bretherick (1986), Furr (1990), Lewis (1992), Lunn and Sansone (1994a), Urben (1995), and Budavari (1996).

[b]Teratogenic chemicals cause developmental malformations; lachrymatory chemicals cause tearing.

only partially or not at all. Prudence dictates that, unless there is good reason for believing otherwise, all chemicals should be regarded as volatile, highly toxic, flammable human carcinogens and should be handled with great care.

Waste should always be disposed of in accordance with all applicable regulations. Waste should be segregated according to institutional requirements, for example, into solid, aqueous, nonchlorinated organic, and chlorinated organic material. Extensive information and cautionary details along with techniques for the disposal of chemicals in laboratories have been published (Bretherick, 1986; Furr, 1990; Aldrich Chemical Co., 1994; Lunn and Sansone, 1994a; Budavari, 1996). Some commonly used disposal procedures are outlined in Basic Protocols 1 to 11. Incorporation of these procedures into laboratory protocols can help to minimize waste disposal problems. Alternate Protocols 1 to 11 describe decontamination methods for some of the chemicals. Support Protocols 1 to 8 describe analytical techniques that are used to verify that reagents have been decontaminated; with modification, these assays may be used to determine the concentration of a particular chemical.

Disposal Methods

A number of procedures for the disposal of hazardous chemicals are available; protocols for the disposal and decontamination of some hazardous chemicals commonly encountered in laboratories are described in this appendix (see Table A.2A.2). These procedures are necessarily brief; for full details consult the original references or a recent collection of these procedures (see Lunn and Sansone, 1994a).

CAUTION: These disposal methods should only be carried out in a chemical fume hood by workers equipped with eye protection, a lab coat, and gloves. Additional protective equipment may be necessary.

Disposal of Benzidine and Diaminobenzidine

BASIC PROTOCOL 1

Benzidine and diaminobenzidine can be degraded by oxidation with potassium permanganate (Castegnaro et al., 1985; Lunn and Sansone, 1991a). This protocol presents a method for decontamination of benzidine and diaminobenzidine in bulk. This method can also be adapted to the decontamination of benzidine and diaminobenzidine spills (see Alternate Protocol 1). These compounds can also be removed from solution using horseradish peroxidase in the presence of hydrogen peroxide (see Alternate Protocol 2).

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Table A.2A.2 Protocols for Disposal of Some Hazardous Chemicals

Protocol	Disposal method for
Basic Protocol 1	Benzidine and diaminobenzidine
Alternate Protocol 1	Spills of benzidine and diaminobenzidine
Alternate Protocol 2	Aqueous solutions of benzidine and diaminobenzidine
Support Protocol 1	Analysis for benzidine and diaminobenzidine
Basic Protocol 2	Silanes
Basic Protocol 3	Cyanide and cyanogen bromide
Support Protocol 2	Analysis for cyanide
Basic Protocol 4	Diisopropyl fluorophosphate in buffer or water
Alternate Protocol 3	Diisopropyl fluorophosphate in *N,N*-dimethylformamide
Alternate Protocol 4	Bulk quantities of diisopropyl fluorophosphate
Support Protocol 3	Spills of diisofluorophosphate
Basic Protocol 5	Ethidium bromide and propidium iodide
Alternate Protocol 5	Equipment contaminated with ethidium bromide
Alternate Protocol 6	Ethidium bromide in isopropanol containing cesium chloride
Alternate Protocol 7	Ethidium bromide in alcohols
Support Protocol 4	Analysis for ethidium bromide and propidium iodide
Basic Protocol 6	Hydrazine
Alternate Protocol 8	Spills of hydrazine
Basic Protocol 7	Hydrogen peroxide
Basic Protocol 8	Iodine
Basic Protocol 9	Mercury compounds
Alternate Protocol 9	Waste water containing mercury
Support Protocol 5	Analysis for mercury
Basic Protocol 10	PMSF in buffer
Alternate Protocol 10	PMSF in isopropanol or dimethyl sulfoxide
Alternate Protocol 11	Bulk quantities of PMSF
Support Protocol 6	Analysis for PMSF
Basic Protocol 11	Sodium azide
Support Protocol 7	Analysis for sodium azide
Support Protocol 8	Analysis for nitrite

Destruction and decontamination are >99%. Support Protocol 1 is used to test for the presence of benzidine and diaminobenzidine.

Materials

Benzidine or diaminobenzidine tetrahydrochloride dihydrate
0.1 M HCl
0.2 M potassium permanganate (prepare immediately before use)
2 M sulfuric acid
Sodium metabisulfite
10 M potassium hydroxide (KOH)

Additional reagents and equipment for testing for the presence of aromatic amines (see Support Protocol 1)

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1. For each 9 mg benzidine, add 10 ml of 0.1 M HCl *or* for each 9 mg of diaminobenzidine tetrahydrochloride dihydrate, add 10 ml water. Stir the solution until the aromatic amine has completely dissolved.
2. For each 10 ml of solution, add 5 ml of 0.2 M potassium permanganate and 5 ml of 2 M sulfuric acid. Allow the mixture to stand for ≥10 hr.
3. Add sodium metabisulfite until the solution is decolorized.
4. Add 10 M KOH to make the solution strongly basic, pH >12.

   CAUTION: *This reaction is exothermic.*
5. Dilute with 5 vol water and filter through filter paper to remove manganese compounds.
6. Test the filtrate for the presence of benzidine or diaminobenzidine (see Support Protocol 1).
7. Neutralize the filtrate with acid and discard.

Decontamination of Spills Involving Benzidine and Diaminobenzidine

ALTERNATE PROTOCOL 1

Additional Materials (also see Basic Protocol 1)

Glacial acetic acid
1:1 (v/v) 0.2 M potassium permanganate/2 M sulfuric acid (prepare immediately before use)

Absorbent material (e.g., paper towels or Kimwipes)
High-efficiency particulate air (HEPA) vacuum (Fisher)

Additional reagents and equipment for testing for the presence of aromatic amines (see Support Protocol 1)

CAUTION: This procedure may damage painted surfaces and Formica.

1. Remove as much of the spill as possible using absorbents and high-efficiency particulate air (HEPA) vacuuming.
2. Wet the surface with glacial acetic acid until all the amines are dissolved, then add an excess of 1:1 (v/v) 0.2 M potassium permanganate/2 M sulfuric acid to the spill area. Allow the mixture to stand ≥10 hr.
3. Ventilate the area and decolorize with sodium metabisulfite.
4. Mop up the liquid with paper towels. Squeeze the solution out of the towels. Discard towels as hazardous solid waste.
5. Add 10 M KOH to basify the solution.

   CAUTION: *This reaction is exothermic.*
6. Dilute with 5 vol water and filter through filter paper to remove manganese compounds.
7. Test the filtrate for the presence of benzidine or diaminobenzidine (see Support Protocol 1).
8. Neutralize the filtrate with acid and discard it.
9. Verify complete decontamination by wiping the surface with a paper towel moistened with water and squeezing the liquid out of the towel. Test the liquid for the presence of benzidine or diaminobenzidine (see Support Protocol 1).

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ALTERNATE PROTOCOL 2

Decontamination of Aqueous Solutions of Benzidine and Diaminobenzidine

The enzyme horseradish peroxidase catalyzes the oxidation of the amine to a radical and the radical diffuses into solution and polymerizes. The polymers are insoluble and fall out of solution.

Additional Materials (also see Basic Protocol 1)

Aqueous solution of benzidine or diaminobenzidine
3% (v/v) hydrogen peroxide
Horseradish peroxidase (see recipe)
1:1 (v/v) 0.2 M potassium permanganate/2 M sulfuric acid
5% (w/v) ascorbic acid
Porous glass filter *or* Sorvall GLC-1 centrifuge or equivalent

Additional reagents and equipment for testing for the presence of aromatic amines (see Support Protocol 1)

1. Adjust the pH of the aqueous benzidine or diaminobenzidine solution to 5 to 7 with acid or base and dilute so the concentration of aromatic amines is ≤100 mg/liter.
2. For each liter of solution, add 3 ml of 3% hydrogen peroxide and 300 U horseradish peroxidase. Let the mixture stand 3 hr.
3. Remove the precipitate by filtering the solution through a porous glass filter or by centrifuging 5 min, room temperature, to pellet the precipitate.

   The precipitate is mutagenic and should be treated as hazardous waste.
4. Immerse the porous glass filter in 1:1 (v/v) 0.2 M potassium permanganate/2 M sulfuric acid.
5. For each liter of filtrate, add 100 ml of 5% ascorbic acid.
6. Test the filtrate for the presence of aromatic amines (see Support Protocol 1).
7. Discard the decontaminated filtrate.

SUPPORT PROTOCOL 1

Analytical Procedures to Detect Benzidine and Diaminobenzidine

Reversed-phase HPLC (*UNIT 11.6*) is used to test for the presence of aromatic amines. The limit of detection is 1 µg/ml for benzidine and 0.25 µg/ml for diaminobenzidine.

Materials

Filtrate from decontaminated aromatic amine (see Basic Protocol 1, Alternate Protocol 1, or Alternate Protocol 2)
10:30:20 (v/v/v) acetonitrile/methanol/1.5 mM potassium phosphate buffer (benzidine) *or* 75:25 (v/v) methanol/1.5 mM potassium phosphate buffer (diaminobenzidine)
1.5 mM potassium phosphate buffer: equimolar amounts of 1.5 mM dibasic potassium phosphate and 1.5 mM monobasic potassium phosphate
250-mm × 4.6-mm-i.d. Microsorb C-8 reversed-phase HPLC column (Rainin Instrument) or equivalent

Additional reagents and equipment for reversed-phase liquid chromatography (*UNIT 11.6*)

Analyze the solution by reversed-phase HPLC using a 250-mm × 4.6-mm-i.d. Microsorb C-8 column. To detect benzidine, elute with 10:30:20 (v/v/v) acetonitrile/methanol/1.5 mM potassium phosphate buffer at a flow rate of 1.5 ml/min and with UV detection at 285 nm. To detect diaminobenzidine, elute with 75:25 (v/v) methanol/1.5 mM potassium phosphate buffer at a flow rate of 1 ml/min and with UV detection at 300 nm.

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Disposal of Chlorotrimethylsilane and Dichlorodimethylsilane

BASIC PROTOCOL 2

Silane-containing compounds are hydrolyzed to hydrochloric acid and polymeric silicon-containing material (Patnode and Wilcock, 1946).

1. Hydrolyze silane-containing compounds by cautiously adding 5 ml silane to 100 ml vigorously stirred water in a flask. Allow the resulting suspension to settle.
2. Remove any insoluble material by filtration and discard it with the solid or liquid hazardous waste.
3. Neutralize the aqueous layer with base and discard it.

Disposal of Cyanides and Cyanogen Bromide

BASIC PROTOCOL 3

Inorganic cyanides (e.g., NaCN) and cyanogen bromide (CNBr) are oxidized by sodium hypochlorite (NaOCl; e.g., Clorox) in basic solution to the much less toxic cyanate ion (Lunn and Sansone, 1985). Destruction is >99.7%.

Materials

Cyanide (e.g., NaCN) or cyanogen bromide (CNBr)
1 M NaOH
5.25% (v/v) sodium hypochlorite (NaOCl; e.g., Clorox bleach)

Additional reagents and equipment for testing for the presence of cyanide (see Support Protocol 2)

1. Dissolve cyanide in water to give a concentration ≤25 mg/ml *or* dissolve CNBr in water to give a concentration ≤60 mg/ml.

   If necessary dilute aqueous solutions so the concentration of NaCN or CNBr does not exceed the limit.

2. Mix 1 vol NaCN or CNBr solution with 1 vol of 1 M NaOH and 2 vol fresh 5.25% NaOCl. Stir the mixture 3 hr.

   IMPORTANT NOTE: *With age Clorox bleach may become ineffective; use of fresh bleach is strongly recommended.*

3. Test the reaction mixture for the presence of cyanide (see Support Protocol 2).
4. Neutralize the reaction mixture and discard it.

Analytical Procedure to Detect Cyanide

SUPPORT PROTOCOL 2

This protocol is used to detect cyanide or cyanogen bromide at ≥3 µg/ml.

Materials

Cyanide or cyanogen bromide decontamination reaction mixture (see Basic Protocol 3)
Phosphate buffer (see recipe)
Cyanide detection reagent (see recipe)
10 mg/ml sodium ascorbate (prepare fresh daily)
100 mg/liter NaCN (prepare fresh weekly)
10 mg/ml chloramine-T (prepare fresh daily)
Sorvall GLC-1 centrifuge or equivalent

1. If necessary to remove suspended solids, centrifuge two 1-ml aliquots of the reaction mixture 5 min at room temperature. Add each supernatant to 4 ml phosphate buffer.
2. If an orange or yellow color appears, add 10 mg/ml sodium ascorbate dropwise until the mixture is colorless, but do not add more than 2 ml.

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3. Add 200 µl of 100 mg/liter NaCN to one reaction mixture.
4. Add 1 ml of 10 mg/ml chloramine-T to each tube. Shake the tubes and let them stand 1 to 2 min.
5. Add 1 ml cyanide detection reagent, shake, and let stand 5 min.

   A blue color indicates the presence of cyanide. If destruction has been complete and the analytical procedure has been carried out correctly, the untreated reaction mixture should be colorless and the control solution, which contains NaCN, should be blue.
6. If necessary to remove suspended solids, centrifuge tubes 5 min at room temperature. Measure the absorbance at 605 nm with appropriate standards and blanks.

BASIC PROTOCOL 4

Disposal of Diisopropyl Fluorophosphate in Buffer or Water

Diisopropyl fluorophosphate can be degraded by adding 1 M NaOH to solutions of DFP in buffer, water (see Basic Protocol 4), or *N,N*-dimethylformamide (DMF; see Alternate Protocol 3) and to bulk quantities of DFP (see Alternate Protocol 4; Lunn and Sansone, 1994a,d). Decontaminating spills is also described (see Support Protocol 3). The product is diisopropylphosphate. The pH should be ≥12, and destruction is >99.8%.

Materials

Solutions of diisopropyl fluorophosphate in buffer or water
1 M NaOH

1. To each 1 ml of 10 mM DFP in buffer or water add 200 ml of 1 M NaOH and check that the reaction mixture is strongly basic (pH ≥12).
2. Allow the reaction mixture to stand 18 hr at room temperature, analyze for completeness of destruction, neutralize, and discard it.

   An analytical procedure for DFP, based on its inhibitory effect on the activity of chymotrypsin, is quite involved, and the reader should refer to Lunn and Sansone (1994a,d) for full details.

   The choice of neutralizing agent is not critical. Acetic acid works well, but any acid may be used.

ALTERNATE PROTOCOL 3

Disposal of Diisopropyl Fluorophosphate in *N,N*-Dimethylformamide

Additional Materials (also see Basic Protocol 4)

Solutions of diisopropyl fluorophosphate in *N,N*-dimethylformamide

1. To each 1 ml of 200 mM DFP in DMF add 2 ml of 1 M NaOH and check that the reaction mixture is strongly basic (pH ≥12).
2. Allow the reaction mixture to stand 18 hr at room temperature, analyze for completeness of destruction, neutralize, and discard it.

   See Lunn and Sansone (1994a,d) for full details on analyzing for DFP activity.

ALTERNATE PROTOCOL 4

Disposal of Bulk Quantities of Diisopropyl Fluorophosphate

Additional Materials (also see Basic Protocol 4)

Bulk quantities of diisopropyl fluorophosphate

1. To each 40 ml of pure DFP add 1 ml of 1 M NaOH and check that the reaction mixture is strongly basic (pH ≥12).

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2. Stir the reaction mixture 1 hr at room temperature, analyze for completeness of destruction, neutralize, and discard it.

See Lunn and Sansone (1994a,d) for full details on analyzing for DFP activity.

Decontamination of Spills Involving Diisopropyl Fluorophosphate

SUPPORT PROTOCOL 3

Additional Materials (also see Basic Protocol 4)

Spills or equipment contaminated with diisopropyl fluorophosphate

1. Add at least 1 ml of 1 M NaOH for each 40 ml of DFP that is estimated to be present. Add more NaOH if required to thoroughly wet all contaminated surfaces. Check that the reaction mixture is strongly basic (pH ≥12) and make sure that all the oily DFP has dissolved.
2. Allow to stand at least 2 hr and preferably 18 hr at room temperature, analyze the solution for completeness of destruction, neutralize, and discard it.

Decontamination is more efficient if it is possible to agitate or stir the reaction mixture (e.g., when the inside of a flask is being decontaminated).

3. Clean the equipment or the spill area in a conventional fashion with soap and water.

Disposal of Ethidium Bromide and Propidium Iodide

BASIC PROTOCOL 5

Ethidium bromide and propidium iodide in water and buffer solutions may be degraded by reaction with sodium nitrite and hypophosphorous acid in aqueous solution (Lunn and Sansone, 1987). Destruction is >99.87%. This reaction may also be used to decontaminate equipment contaminated with ethidium bromide (see Alternate Protocol 5; Lunn and Sansone, 1989) and to degrade ethidium bromide in organic solvents (see Alternate Protocol 6 and Alternate Protocol 7; Lunn and Sansone, 1990). Ethidium bromide is frequently extracted from nucleic acids using organic solvents. Ethidium bromide and propidium iodide may also be removed from aqueous solution by adsorption onto Amberlite XAD-16 resin.

Materials

Ethidium bromide– or propidium iodide–containing solution in water, buffer, or 1 g/ml cesium chloride
5% (v/v) hypophosphorous acid (prepare fresh daily by diluting commercial 50% reagent 1/10)
0.5 M sodium nitrite (prepare fresh daily)
Sodium bicarbonate
Additional reagents and equipment for testing for the presence of ethidium bromide or propidium iodide (see Support Protocol 4)

1. If necessary, dilute the ethidium bromide– or propidium iodide–containing solution so the concentration of ethidium bromide or propidium iodide is ≤0.5 mg/ml.
2. For each 100 ml solution, add 20 ml of 5% hypophosphorous acid solution and 12 ml of 0.5 M sodium nitrite. Stir briefly and let stand 20 hr.
3. Neutralize the reaction mixture by adding sodium bicarbonate until the evolution of gas ceases.
4. Test the reaction mixture for the presence of ethidium bromide or propidium iodide (see Support Protocol 4).
5. Discard the decontaminated reaction mixture.

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ALTERNATE PROTOCOL 5

Decontamination of Equipment Contaminated with Ethidium Bromide

Glass, stainless steel, Formica, floor tile, and the filters of transilluminators have been successfully decontaminated using this protocol. No change in the optical properties of the transilluminator filter could be detected, even after a number of decontamination cycles.

Materials

Equipment contaminated with ethidium bromide
Decontamination solution: 0.2 M sodium nitrite/3.3% (v/v) hypophosphorous acid (prepare just before use)
Sodium bicarbonate

Additional reagents and equipment for testing for the presence of ethidium bromide (see Support Protocol 4)

1. Wash the equipment once with a paper towel soaked in decontamination solution.

   The pH of the decontamination solution is 1.8. If this would be too corrosive for the surface to be decontaminated, wash the surface with a paper towel soaked in water.

2. Wash the surface five times with paper towels soaked in water, using a fresh towel each time.
3. Soak all the towels 1 hr in decontamination solution.
4. Neutralize the decontamination solution by adding sodium bicarbonate until the evolution of gas ceases.
5. Test the decontamination solution for the presence of ethidium bromide (see Support Protocol 4).
6. Discard the decontamination solution and the paper towels as nonhazardous liquid and solid wastes.

ALTERNATE PROTOCOL 6

Decontamination of Ethidium Bromide in Isopropanol Saturated with Cesium Chloride

Materials

Ethidium bromide in isopropanol saturated with cesium chloride
Decontamination solution: 0.2 M sodium nitrite/3.3% (v/v) hypophosphorous acid (prepare just before use)
Sodium bicarbonate

Additional reagents and equipment for testing for the presence of ethidium bromide (see Support Protocol 4)

1. If necessary, dilute the ethidium bromide/isopropanol/cesium chloride so the concentration of ethidium bromide is ≤1 mg/ml.
2. For each volume of ethidium bromide solution, add 4 vol decontamination solution. Stir the reaction mixture 20 hr.
3. Neutralize the reaction mixture by adding sodium bicarbonate until the evolution of gas ceases.
4. Test the reaction mixture for the presence of ethidium bromide (see Support Protocol 4).
5. Discard the decontaminated solution.

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Decontamination of Ethidium Bromide in Isoamyl Alcohol and 1-Butanol

ALTERNATE PROTOCOL 7

Materials

Ethidium bromide in isoamyl alcohol or 1-butanol
Decontamination solution: 0.2 M sodium nitrite/3.3% (v/v) hypophosphorous acid (prepare just before use)
Activated charcoal
Sodium bicarbonate
Separatory funnel

Additional reagents and equipment for testing for the presence of ethidium bromide (see Support Protocol 4)

1. If necessary, dilute the ethidium bromide/isoamyl alcohol or ethidium bromide/1-butanol so the concentration of ethidium bromide is ≤1 mg/ml.
2. For each volume of ethidium bromide solution, add 4 vol decontamination solution. Stir the two-phase reaction mixture rapidly for 72 hr.
3. For each 100 ml of reaction mixture, add 2 g activated charcoal. Stir another 30 min.
4. Filter the reaction mixture.
5. Neutralize the filtrate by adding sodium bicarbonate until the evolution of gas ceases. Separate the layers using a separatory funnel.

   More alcohol may tend to separate from the aqueous layer on standing.
6. Test the alcohol and aqueous layers for the presence of ethidium bromide (see Support Protocol 4).
7. Discard the alcohol and aqueous layers appropriately. Discard the activated charcoal as solid waste.

   The aqueous layer contains 4.6% 1-butanol or 2.3% isoamyl alcohol.

Analytical Procedure to Detect Ethidium Bromide or Propidium Iodide

SUPPORT PROTOCOL 4

This protocol is used to verify that solutions no longer contain ethidium bromide or propidium iodide. The limits of detection are 0.05 parts per million (ppm) for ethidium bromide and 0.1 ppm for propidium iodide.

Materials

Reaction mixture containing ethidium bromide or propidium iodide (see Basic Protocol 5 or Alternate Protocol 5, 6, or 7)
TBE electrophoresis buffer, pH 8.1 (see recipe)
20 µg/ml calf thymus DNA in TBE electrophoresis buffer, pH 8.1
Spectophotofluorometer *or* hand-held UV lamp

1. Mix 100 µl reaction mixture containing ethidium bromide or propidium iodide with 900 µl TBE electrophoresis buffer.
2. Add 1 ml of 20 µg/ml calf thymus DNA. Prepare a blank solution (100 µl water + 900 µl TBE electrophoresis buffer + 1 ml of 20 µg/ml calf thymus DNA) and control solutions containing known quantities of ethidium bromide or propidium iodide. Let the mixtures stand 15 min.
3. To detect ethidium bromide, measure the fluorescence with an excitation wavelength of 540 nm and an emission wavelength of 590 nm. To detect propidium iodide,

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measure the fluorescence with an excitation wavelength of 350 nm and an emission wavelength of 600 nm.

If a spectrophotofluorometer is not available, fluorescence of ethidium bromide can be qualitatively determined using a hand-held UV lamp on the long-wavelength setting (Lunn and Sansone, 1991c).

BASIC PROTOCOL 6

Disposal of Hydrazine

Hydrazine may be oxidized with potassium permanganate in sulfuric acid (Castegnaro et al., 1986). Destruction is >99.997%. This method can also be adapted for the decontamination of spills of hydrazine (see Alternate Protocol 8). The limit of detection for hydrazine is 10 µg/liter.

CAUTION: This procedure works only for hydrazine itself. Oxidation of methylated hydrazines may result in the generation of carcinogenic nitrosamines and should not be attempted. However, hydrazine and methylated hydrazines can be reduced with nickel-aluminum alloy without producing nitrosamines (Lunn et al., 1983).

Materials

Hydrazine
18.3 M (concentrated) sulfuric acid
Potassium permanganate
Sodium metabisulfite
10 M potassium hydroxide (KOH)
1 M HCl
Hydrazine detection reagent (see recipe)

Perform decontamination

1. Dissolve 25 mg hydrazine in 10 ml water.
2. Add 1 ml concentrated sulfuric acid.

   CAUTION: *This reaction is exothermic.*
3. Add 290 mg potassium permanganate. Stir 16 hr.
4. At the end of the reaction, decolorize the solution with sodium metabisulfite.
5. Make the solution strongly basic by adding 10 M KOH to pH >12.

   CAUTION: *This reaction is exothermic.*
6. Dilute the reaction mixture with 5 vol water.
7. Filter through filter paper to remove manganese compounds.

Analyze the filtrate for hydrazine

8. Neutralize the filtrate with acid.
9. Add 2-ml filtrate to 2 ml of 1 M HCl and 2 ml hydrazine detection reagent. Measure the absorbance at 458 nm against a suitable blank to test for the presence of hydrazine.
10. Discard the decontaminated filtrate.

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Decontamination of Spills of Hydrazine

ALTERNATE PROTOCOL 8

This protocol is used to decontaminate spills of hydrazine. It may damage painted surfaces or Formica.

Additional Materials (also see Basic Protocol 6)

3 M sulfuric acid
Potassium permanganate solution: 0.3 M potassium permanganate (47.4 g/liter) in 3 M sulfuric acid (prepare fresh)
Absorbent (e.g., paper towels or Kimwipes)

Decontaminate the spill

1. Add 3 M sulfuric acid to the spill and remove as much of the spill as possible with an absorbent.
2. Place the absorbent material in an appropriate solvent and decontaminate it (see Basic Protocol 6).
3. Cover the spill area with potassium permanganate solution. Let the mixture stand overnight.
4. Decolorize the mixture with sodium metabisulfite. Remove the solution with absorbent.

Analyze absorbed liquid

5. Squeeze out the absorbent. Discard absorbent as hazardous solid waste.
6. Make the solution strongly basic with 10 M KOH.

   CAUTION: *This reaction is exothermic.*
7. Dilute the solution with 5 vol water.
8. Filter the solution through filter paper to remove manganese compounds.
9. Neutralize the filtrate.
10. Add 2-ml filtrate to 2 ml of 1 M HCl and 2 ml hydrazine detection reagent. Measure absorbance at 458 nm against a suitable blank to test for the presence of hydrazine.
11. Discard the decontaminated filtrate.
12. Verify decontamination by taking wipe samples of the area using pieces of absorbent moistened with water to wipe over the area. Squeeze the water out of the absorbent and test for hydrazine as described in step 10.
13. Clean the surface in a conventional manner with soap and water.

Disposal of Hydrogen Peroxide

BASIC PROTOCOL 7

Hydrogen peroxide can be reduced with sodium metabisulfite (Lunn and Sansone, 1994a).

Materials

30% hydrogen peroxide
10% (w/v) sodium metabisulfite
10% (w/v) potassium iodide
1 M HCl
1% (w/v) starch indicator solution

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1. Add 5 ml of 30% hydrogen peroxide to 100 ml of 10% sodium metabisulfite. Stir the mixture at room temperature until the temperature starts to drop, indicating that the reaction is over.

2. Test for the presence of hydrogen peroxide by adding a few drops of the reaction mixture to an equal volume of 10% potassium iodide. Add a few drops of 1 M HCl to acidify the reaction mixture, then add a drop of 1% starch indicator solution.

   A deep blue color indicates the presence of excess oxidant. If necessary, add more 10% sodium metabisulfite until the starch test is negative.

3. Discard the decontaminated mixture.

BASIC PROTOCOL 8

Reduction of Iodine

Iodine is reduced with sodium metabisulfite to iodide (Lunn and Sansone, 1994b).

Materials

Iodine
10% (w/v) sodium metabisulfite
1 M HCl
1% (w/v) starch indicator solution

1. Add 5 g iodine to 100 ml of 10% sodium metabisulfite. Stir the mixture until the iodine has completely dissolved.

2. Acidify a few drops of the reaction mixture by adding a few drops of 1 M HCl. Add 1 drop of 1% starch indicator solution.

   A deep blue color indicates the presence of iodine. If reduction is not complete, add more sodium metabisulfite solution.

3. Dispose of the decontaminated solution.

BASIC PROTOCOL 9

Disposal of Mercury Compounds

Solutions of mercuric acetate can be decontaminated using Dowex 50X8-100, a strongly acid gel-type ion-exchange resin with a sulfonic acid functionality. Solutions of mercuric chloride can be decontaminated using Amberlite IRA-400(Cl), a strongly basic gel-type ion-exchange resin with a quaternary ammonium functionality. The final concentration of mercury is <3.8 ppm (Lunn and Sansone, 1994a). On a small scale it is most convenient to stir the resin in the solution to be decontaminated, but on a larger scale, or for routine use, it may be more convenient to pass the solution through a column packed with the resin. Although the volume of waste that must be disposed of is greatly reduced using this technique, a small amount of waste (i.e., the resin contaminated with mercury) remains and must be discarded appropriately. Resin can be regenerated by washing with acid, but the concentrated metal-containing solution generated by this must also be disposed of appropriately. Mercury may also be removed from laboratory waste water using a column of iron powder (see Alternate Protocol 9). Support Protocol 5 is used to detect the presence of mercury.

Materials

Solution containing mercuric acetate *or* mercuric chloride
Dowex 50X8-100 ion-exchange resin *or* Amberlite IRA-400 (Cl) ion-exchange resin

Additional reagents and equipment to test for the presence of mercury (see Support Protocol 5)

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1a. *For mercuric acetate:* For each 200 ml of solution containing ≤1600 ppm mercuric acetate, add 1 g Dowex 50X8-100 ion-exchange resin. Stir the mixture 1 hr, then filter through filter paper.

1b. *For mercuric chloride:* For each 200 ml of solution containing ≤1350 ppm mercuric chloride, add 1 g Amberlite IRA-400(Cl) ion-exchange resin. Stir the mixture 6 hr, then filter through filter paper.

The speed and efficiency of decontamination will depend on factors such as the size and shape of the flask and the rate of stirring.

3. Test the filtrate for the presence of mercury (see Support Protocol 5).

4. Discard the decontaminated filtrate and the mercury-containing resin appropriately.

Decontamination of Waste Water Containing Mercury

ALTERNATE PROTOCOL 9

Laboratory waste water that contains mercury is decontaminated by passing it through a column of iron powder. The mercury forms mercury amalgam and stays on the column. Some metallic mercury remains in solution but this can be removed by aeration. The final concentration of mercury is <5 ppb (Shirakashi et al., 1986).

Materials

Waste water containing mercury
Iron powder, 60 mesh
6-mm-i.d. column

1. Pack a 6-mm-i.d. column with 1 g of 60-mesh iron powder.

Use a fresh column for each treatment.

2. Pass ≤2 liters of water containing ≤2.5 ppm of mercury through the column at a flow rate of 250 ml/hr.

Solutions containing a higher concentration of mercury may also be treated, but this will result in a higher final concentration of mercury (e.g., treating a 100-ppm solution in this fashion yielded 33 ppb final).

Some iron ends up in solution and can be removed by adjusting the pH to 8. Precipitated $Fe(OH)_3$ can be removed by filtration.

3. Aerate the resulting effluent to remove traces of metallic mercury and continue aeration 30 min after the last of the effluent has emerged from the column. Vent the metallic mercury removed from the solution by aeration into the fume hood or capture it in a mercury trap.

The effluent contains <5 ppb mercury. The presence of iodide or polypeptone may necessitate several treatments to reduce the mercury to an acceptable level.

Analytical Procedure to Detect Mercury

SUPPORT PROTOCOL 5

Atomic absorption spectroscopy with detection at 253.7 nm, a slit width of 0.7 nm, and a limit of detection of 3.8 ppm can be used to determine the concentration of mercury in solution for experiments involving ion-exchange resins. A Hiranuma mercury meter model HG-1 can be used for experiments involving iron powder.

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BASIC PROTOCOL 10

Disposal of PMSF in Buffer

Phenylmethylsulfonyl fluoride (PMSF) may be degraded by making the buffer solution strongly alkaline (pH ≥12) with 1 M NaOH solution. Stock solutions of PMSF in isopropanol or DMSO can also be degraded by adding 1 M NaOH (see Alternate Protocol 10; Lunn and Sansone, 1994a,c); bulk quantities of PMSF are dissolved in isopropanol before being neutralized (see Alternate Protocol 11). Degradation is >99.9%. A method of detecting PMSF via RP-HPLC is also discussed (see Support Protocol 6).

Materials

Solutions of PMSF in buffer
1 M NaOH
Acetic acid, glacial

Additional reagents and equipment for analyzing PMSF destruction (see Support Protocol 6)

1. For each 10 ml of buffer containing up to 10 mM PMSF, add 1 ml of 1 M NaOH solution. Ensure that the pH is ≥12.
2. After 1 hr neutralize with 1 ml of acetic acid, test for completeness of destruction (see Support Protocol 6), and discard the reaction mixture.

ALTERNATE PROTOCOL 10

Disposal of PMSF in Isopropanol or Dimethyl Sulfoxide

Additional Materials (also see Basic Protocol 10)

Solutions of PMSF in isopropanol or dimethyl sulfoxide

1. Dilute the solution, if necessary, so that the concentration of PMSF does not exceed 100 mM.
2. For each 1 ml of solution add 5 ml of 1 M NaOH, shake to ensure complete mixing, and allow to stand for 24 hr.
3. Neutralize with 1 ml of acetic acid, test for completeness of destruction (see Support Protocol 6), and discard the reaction mixture.

ALTERNATE PROTOCOL 11

Disposal of Bulk Quantities of PMSF

Additional Materials (also see Basic Protocol 10)

Bulk quantities of PMSF

1. Dissolve the PMSF in isopropanol so that the concentration does not exceed 100 mM.

   Stirring may be necessary.
2. For each 1 ml of solution add 5 ml of 1 M NaOH, shake to ensure complete mixing, and allow to stand for 24 hr.
3. Neutralize with 1 ml of acetic acid, test for completeness of destruction (see Support Protocol 6), and discard the reaction mixture.

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Analytical Procedure to Detect PMSF

SUPPORT PROTOCOL 6

Materials

Decontaminated and neutralized reaction mixtures
50:50 (v/v) acetonitrile/water
250-mm × 4.6-mm-i.d. Microsorb C-8 reversed-phase HPLC column (Rainin Instrument) or equivalent

Analyze decontaminated and neutralized reaction mixtures by reversed-phase HPLC (RP-HPLC; *UNIT 11.6*) using a 250-mm × 4.6-mm-i.d. Microsorb C-8 column and eluting with 50:50 (v/v) acetonitrile/water at a flow rate of 1 ml/min with UV detection at 220 nm.

Disposal of Sodium Azide

BASIC PROTOCOL 11

Sodium azide can be oxidized by ceric ammonium nitrate (Manufacturing Chemists Association, 1973) to nitrogen (Mason, 1967) or by nitrous acid (National Research Council, 1983) to nitrous oxide (Mason, 1967). Destruction is >99.996%. Sodium azide in buffer solution may also be degraded by the addition of sodium nitrite (Lunn and Sansone, 1994a). The reaction proceeds much more readily at low pH, but if sufficient sodium nitrite is added, it will proceed to completion even at high pH. At low pH, it may be possible to completely degrade the azide present in the buffer with less than the amount of sodium nitrite indicated. However, the reaction mixture must be carefully checked to ensure that no azide remains (see Support Protocol 7). At high pH it is possible for unreacted azide to remain in the presence of excess nitrite. Residual nitrite can be detected using Support Protocol 8.

CAUTION: Some toxic nitrogen dioxide may be produced as a by-product of these reactions, so they should always be carried out in a chemical fume hood.

Materials

Sodium azide or solution containing sodium azide
Ceric ammonium nitrate
10% (w/v) potassium iodide
1 M HCl
1% (w/v) starch indicator solution
Sodium nitrite
4 M sulfuric acid
Litmus paper

Additional reagents and equipment to test for the presence of sodium azide (see Support Protocol 7) *or* nitrite (see Support Protocol 8)

Decontamination using ceric ammonium nitrate

1a. For each gram of sodium azide, add 9 g ceric ammonium nitrate to 30 ml of water, and stir until it has dissolved.

2a. Dissolve each gram of sodium azide in 5 ml water. Add this solution to the ceric ammonium nitrate solution at the rate of 1 ml each min. Stir 1 hr.

If the reaction is carried out on a larger scale, an ice bath may be required for cooling.

3a. Check that the reaction is still oxidizing by adding a few drops of the reaction mixture to an equal volume of 10% potassium iodide. Acidify the mixture with 1 drop of 1 M HCl. Add 1 drop of 1% starch indicator solution.

The deep blue color of the starch-iodine complex indicates that excess oxidant is present. If excess oxidant is not present, add more ceric ammonium nitrate.

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4a. Test for the presence of sodium azide (see Support Protocol 8).

5a. Discard the decontaminated reaction mixture.

Decontamination using sodium nitrite

1b. For each 5 g sodium azide, dissolve 7.5 g sodium nitrite in 30 ml water.

2b. Dissolve each 5 g sodium azide in 100 ml water. Add the sodium nitrite solution with stirring. Slowly add 4 M sulfuric acid until the reaction mixture is acidic to litmus. Stir 1 hr.

CAUTION: *It is important to add the sodium nitrite, then the sulfuric acid. Adding these reagents in reverse order will generate explosive, volatile, toxic hydrazoic acid.*

If the reaction is carried out on a large scale, an ice bath may be required for cooling.

3b. Check that there is excess nitrous acid in the reaction. Add a few drops of the reaction mixture to an equal volume of 10% potassium iodide. Acidify the mixture with 1 drop of 1 M HCl. Add 1 drop starch indicator solution.

The deep blue color of the starch-iodine complex indicates that excess nitrous acid is present. If excess nitrous acid is not present, add more sodium nitrite.

4b. If excess nitrous acid is present, test for the presence of sodium azide (see Support Protocol 7).

5b. Discard the decontaminated reaction mixture.

Decontamination of sodium azide in buffer

1c. If necessary, dilute the buffer solution with water so the concentration of sodium azide is ≤1 mg/ml.

2c. For each 50 ml buffer solution add 5 g sodium nitrite. Stir the reaction 18 hr.

3c. Test for the presence of sodium azide (see Support Protocol 7).

4c. Discard the decontaminated reaction solution.

SUPPORT PROTOCOL 7

Analytical Procedures to Detect Sodium Azide

Sodium azide is analyzed by reacting azide ion with 3,5-dinitrobenzoyl chloride to form 3,5-dinitrobenzoyl azide, which can be detected by reversed-phase HPLC. The limit of detection of this assay is 0.2 µg/ml sodium azide. This protocol works only in the absence of nitrite; Support Protocol 8 should be used to verify that all of the nitrite has been destroyed by sulfamic acid.

Materials

Reaction mixture from sodium azide treated with ceric ammonium nitrate or sodium nitrite
1 M KOH
Acetonitrile
Sodium azide indicator solution (see recipe)
0.2 M HCl
20% (w/v) sulfamic acid
3,5-dinitrobenzoyl chloride
50:50 (v/v) acetonitrile/water

Sorvall GLC-1 centrifuge or equivalent
25-cm × 4.6-mm-id Microsorb C-8 reversed-phase HPLC column (Rainin Instruments) or equivalent

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Additional reagents and equipment for reversed-phase liquid chromatography (*UNIT 11.6*)

To analyze for azide in the presence of ceric salts

1a. To a 10-ml aliquot of the reaction mixture from the treatment of sodium azide with ceric ammonium nitrate, add 40 ml water. Add 5 ml of this diluted solution to 3 ml of 1 M KOH and mix by shaking.

If <3 ml of 1 M KOH is used, precipitation of ceric salts will not be complete.

2a. Centrifuge the mixture 5 min, room temperature.

3a. Remove 2 ml supernatant and add to 1 ml acetonitrile. Add 1 drop sodium azide indicator solution, add 0.2 M HCl dropwise until the mixture turns yellow, then add 1 drop more.

To analyze for azide in the presence of nitrite

1b. To 5 ml of the reaction mixture from sodium azide treated with sodium nitrite add ≥1 ml sulfamic acid to remove excess nitrite. Let stand ≥3 min.

More sulfamic acid solution may be required for strongly basic reaction mixtures or those containing high concentrations of nitrite. Complete removal of nitrite can be checked by using a modified Griess reagent (see Support Protocol 8).

At high pH the reaction between azide and nitrite is quite slow, so the presence of excess nitrite does not mean that all the azide has been degraded.

2b. Add 1 drop sodium azide indicator solution, then basify the mixture by adding 1 M KOH until it turns purple (typically, 3 to 10 ml are required).

3b. Add 2 ml acetonitrile. Add 0.2 M HCl dropwise until the mixture turns yellow, then add 1 drop more.

If >1 ml sulfamic acid is used, add 4 ml acetonitrile.

4. Prepare a 10% (w/v) solution of 3,5-dinitrobenzoyl chloride in acetonitrile.

5. Add 50 µl of 10% dinitrobenzoyl chloride/acetonitrile to the reaction mix (step 3a or 3b). Shake the mixture and let it stand ≥3 min.

Longer standing times have no effect on the HPLC analysis. However, it is crucial to use freshly prepared 3,5-dinitrobenzoyl chloride solution within minutes of its preparation. It is generally most convenient to prepare all the analytical samples with the fresh solution at the beginning of the day and analyze them over the course of the day.

6. Analyze 20 µl of each reaction mixture by reversed-phase HPLC using a mobile phase of 50:50 (v/v) acetonitrile/water with a flow rate of 1 ml/min and UV detection at 254 nm.

The peak for 3,5-dinitrobenzoyl azide elutes at ~9 min.

Analytical Procedure to Detect Nitrite

SUPPORT PROTOCOL 8

This protocol uses a modified Griess reagent to test for the presence of nitrite. The limit of detection of this assay is 0.06 µg/ml nitrite. A similar procedure uses *N*-(1-naphthyl)-ethylenediamine (Helrich, 1990).

Materials

Reaction mixture treated to remove excess nitrite (see Support Protocol 7, step 1b)
α-Naphthylamine
15% (v/v) aqueous acetic acid
Sulfanilic acid solution (see recipe)

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1. Prepare the modified Griess reagent: boil 0.1 g α-naphthylamine in 20 ml water until it dissolves. While the solution is still hot, pour it into 150 ml of 15% aqueous acetic acid. Add 150 ml sulfanilic acid solution.

   This reagent should be stored at room temperature in a brown bottle.

   CAUTION: *α-Naphthylamine is a carcinogen.*

2. Add 3 ml of the reaction mixture treated to remove excess nitrite to 1 ml modified Griess reagent. Let stand 6 min at room temperature.

3. Measure the absorbance at 520 nm against a suitable blank.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Cyanide detection reagent

Stir 3.0 g barbituric acid in 10 ml water. Add 15 ml of 4-methylpyridine and 3 ml concentrated HCl while continuing to stir. Cool and dilute to 50 ml with water. Store at room temperature.

CAUTION: *This reaction is exothermic.*

Horseradish peroxidase

Dissolve hydrogen-peroxide oxidoreductase (EC 1.11.1.7 [Type II]; specific activity 150 to 200 purpurogallin U/mg, Sigma) in 1 g/liter sodium acetate to give 30 U/ml. Prepare fresh daily.

For small-scale reactions, a more dilute solution can be used to avoid working with inconveniently small volumes.

Hydrazine detection reagent

1.6 g *p*-dimethylaminobenzaldehyde (0.12 M final)
80 ml ethanol (91% final)
8 ml concentrated HCl (1.05 M final)
Store at room temperature

Phosphate buffer

13.6 g monobasic potassium phosphate (KH_2PO_4; 0.1 M final)
0.28 g dibasic sodium phosphate (Na_2HPO_4; 2 mM final)
3.0 g potassium bromide (KBr; 25 mM final)
1 liter H_2O
Store at room temperature

Potassium bromide is necessary to make the assay for cyanide work correctly.

Sodium azide indicator solution

0.1 g bromocresol purple (0.4% final)
18.5 ml 0.01 M potassium hydroxide (KOH; 7.4 mM final)
H_2O to 25 ml
Store at room temperature

Sulfanilic acid solution

Dissolve 0.5 g sulfanilic acid in 150 ml of 15% (v/v) aqueous acetic acid. Use immediately.

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TBE electrophoresis buffer, pH 8.1

10× stock solution, 1 liter:

108g Tris base (890 mM final)
55 g boric acid (890 nM final)
40 ml 0.5 M EDTA, pH 8.0 (20 mM)
Adjust pH to 8.1

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Safe Use of Radioisotopes

The use of radioisotopes to label specific molecules in a defined way has greatly furthered the discovery and dissection of biochemical pathways. The development of methods to inexpensively synthesize such tagged biological compounds on an industrial scale has enabled them to be used routinely in laboratory protocols, including many detailed in this manual. Although most of these protocols involve the use of only microcurie (μCi) amounts of radioactivity, some (particularly those describing the metabolic labeling of proteins within cells; see *UNIT 3.4*) require amounts on the order of tens of millicuries (mCi). In all cases where radioisotopes are used, depending on the quantity and nature of the isotope, certain precautions must be taken to ensure the safety of the scientist. This appendix outlines a few such considerations relevant to the isotopes most frequently used in biological research.

In designing safe protocols for the use of radioactivity, the importance of common sense, based on an understanding of the general principles of isotopic decay and the importance of continuous monitoring with a hand-held radioactivity monitor (e.g., Geiger counter), cannot be overemphasized. In addition, it is also critical to take into account the rules, regulations, and limitations imposed by each specific institution. These are usually not optional considerations: an institution's license to use radioactivity normally depends on strict adherence to such rules.

Many of the protocols described have evolved (and are evolving still) over the years and through the millicuries in the Department of Molecular Biology and Virology at the Salk Institute. The authors are indebted to those who have trained them in the safe use of radioactivity, in particular to the members of the Salk Institute Radiation Safety Department. Most of the designs for the shields and other safety equipment shown in Figures A.2B.1, A.2.B2, and A.2B.3 were created at the Salk Institute in collaboration with Dave Clarkin and Mario Tengco. Safety equipment of similar design is available from several commercial vendors, including CBS Scientific and Research Products International (see *SUPPLIERS APPENDIX*).

BACKGROUND INFORMATION

The Decay Process

As anyone who has taken a basic chemistry course will remember, each element is characterized by its atomic number, defined as the number of orbital electrons or the number of protons in the nucleus of that atom. Isotopes of a given element exist because some atoms of each element, while by definition having the same number of protons, have a different number of neutrons and therefore a different nuclear weight. It should be noted that the number of electrons outside the nucleus remains the same for all isotopes of a given element, and so all isotopes of a given element are equivalent with respect to their chemical reactivity.

Radioactive decay occurs when subatomic particles are released from the nucleus of an atom of a heavy isotope. This often results in the conversion of an atom of one isotope to an isotope of a different element, because the original isotope's atomic number changes after decay. The subatomic particles released from naturally occurring radioisotopes are of three basic types: α and β particles and γ rays.

An α particle is essentially the nucleus of a helium atom, or two protons plus two neutrons. It is a relatively large, heavy particle that moves slowly and usually only across short distances before it encounters some other atom with which it interacts. These particles are released from isotopes with large nuclei (atomic number >82; e.g., plutonium or uranium); such isotopes are not commonly used in biological research.

In contrast to α particles, β particles are light, high-speed charged particles. Negatively charged β particles are essentially electrons of nuclear origin that are released when a neutron is converted to a proton. Release of a β particle thus changes the atomic number and elemental status of the isotope.

γ radiation has both particle and wave properties; its wavelength falls within the range of X-ray wavelengths. The distinction between γ rays and X rays was made when primitive X-ray machines produced X rays with a wavelength longer than those of the γ rays produced naturally by radioisotopes. Modern X-ray machines produce a much broader spectrum of wavelengths, including γ radiation; currently this sort of X-ray radiation is termed γ when it is of nuclear origin. Unlike β-particle release, the release of γ radiation by itself produces an isotopic change rather than an elemental one; however, the resultant nuclei are unstable and often decay further, releasing β particles.

The energy of all α particles and γ rays (measured in electron volts) is fixed, because

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Table A.2B.1 Physical Characteristics of Commonly Used Radionuclides[a]

Nuclide	Half-life	Emission	Energy, max (MeV)	Range of emission, max	Approx. specific activity at 100% enrichment (Ci/mg)	Atom resulting from decay	Target organ
^{3}H	12.43 years	β	0.0186	0.42 cm (air)	9.6	$^{3}_{2}He$	Whole body
^{14}C	5370 years	β	0.156	21.8 cm (air)	4.4 mCi/mg	$^{14}_{7}N$	Bone, fat
$^{32}P^{b}$	14.3 days	β	1.71	610 cm (air) 0.8 cm (water) 0.76 cm (Plexiglas)	285	$^{33}_{16}S$	Bone
$^{33}P^{b}$	25.4 days	β	0.249	49 cm	156	$^{33}_{16}S$	Bone
^{35}S	87.4 days	β	0.167	24.4 cm (air)	43	$^{35}_{17}Cl$	Testes
$^{125}I^{c}$	60 days	γ	0.27–0.035	0.2 mm (lead)	14.2	$^{125}_{52}Te$	Thyroid
$^{131}I^{c}$	8.04 days	β	0.606	165 cm (air)	123	$^{130}_{54}Xe$	Thyroid
		γ	0.364	2.4 cm (lead)			

[a]Table compiled based on information in Lederer et al. (1967) and Shleien (1987).
[b]Recommended shielding is Plexiglas; half-value layer measurement is 1 cm.
[c]Recommended shielding is lead; half-value layer measurement is 0.02 mm.

they are of specific composition or wavelength. The energy of β particles, however, varies depending on the atom they originate from (and with concomitant release of neutrinos or antineutrinos that serve to balance the conservation of energy aspect of the decay equation). Thus there are (relatively) high-energy β particles released during the decay of ^{32}P and low-energy β particles released when tritium (^{3}H) decays.

Isotopic decay usually involves a chain or sequence of events rather than a single loss of a particle, because the resultant, equally unstable atoms try to achieve equilibrium. During this course of decay, secondary forms of radiation can be generated that may also pose a hazard to workers. For example, when high-energy β particles released during the decay of ^{32}P encounter the nuclei of atoms with a large atomic number, a strong interaction occurs. The β particle loses some energy in the form of a photon. Such photons are called Bremsstrahlung radiation; they are detectable using a monitor suitable for the detection of γ or X rays.

Following their release, α, β, and γ emissions (as well as secondary forms of radiation) travel varying average distances at varying average speeds, depending on their energy and the density of the material through which they are moving. The distance they actually travel before encountering either the electrons or nucleus of another atom is termed their degree of penetrance. This value is expressed as an average for each type of particle. The energy of the particles released (and therefore their potential penetrance) thus dictates what type of shielding, if any, is necessary for protection against the radiation generated by the decay of a given isotope. α, β, and γ emissions all have the potential, upon encountering an atom, to knock out its electrons, thereby creating ions. Thus, these three types of emissions are called ionizing radiation. The formation of such ions may result in the perturbation of biological processes: therein lies the danger associated with radioactivity!

Table A.2B.1 provides a summary of the factors discussed above for several isotopes commonly used in biochemical research.

Measuring Radioactivity and Individual Exposure to It

The radioactivity of a given substance is measured in terms of its ionizing activity. A Curie by definition is the amount of radioactive material that will produce 3.4×10^{10} disintegration (ions) per second. This, not coincidentally, happens to be the number of disintegrations that occur during the decay of one gram of radium and its decay products. Exposure to such radiation is measured as the amount of energy absorbed by the recipient, which, of course, is directly related to the potential damage such radiation may cause. One rad is the dose of radiation that will cause 100 ergs of energy to be absorbed per gram of irradiated material. Another unit commonly used to measure radiation doses is the rem; this is related to the rad but takes into account a "quality

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factor" based on the type of ionizing radiation being received. For β particles and γ or X rays this factor is 1; therefore, rems β equal rads β. In contrast, the quality factor associated with α particles is 20, so an exposure of one rad due to α particles would be recorded as 20 rem.

The amount or dose of radiation received by materials (cells, scientists, etc.) near the source depends not only on the specific type and energy (penetrance) of the radiation being produced, but also on the subject's distance from the source, the existence of any intervening layers of attenuating material (shielding), and the length of time spent in the vicinity of the radiation source. To best measure such doses, every person working with or around radioactivity should wear an appropriate type of radiation detection badge (in addition to carrying a portable radiation monitor that can give an immediate, approximate reading.) This is normally a requirement (not an option) for compliance with an institution's license to use radioisotopes. Such badges are usually furnished by the safety department and collected at regular intervals for reading by a contracted company. At most institutions, the old-style film badges have been replaced with the more accurate TLDs (thermoluminescent dosimeters). These take advantage of chemicals such as calcium or lithium fluorides which, following exposure to ionizing radiation, will luminesce at temperatures below their normal thermal luminescence threshold. Different types of badges are sensitive to different types of radiation: always be sure to wear one that is appropriate for detecting exposure to the isotope being used! In most places pregnant women are asked to wear a more sensitive (and more expensive) dosimeter to better monitor their (and the developing fetus') exposure. Most often workers will be asked to wear a radiation detection badge on the labcoat lapel in order to measure whole-body radiation. When working with ^{32}P or ^{125}I it is also advisable to wear a ring badge to measure exposure to the unshielded extremities (fingers). The limit set for "acceptable" exposure to whole-body radiation is several-fold less than the limit set for extremities. Nevertheless, we have found that the exposure recorded on ring badges is often significant with respect to the limit for extremities set by our institution.

What is known about the effects on humans of exposure to low levels of radiation (i.e., levels which would be received when briefly handling mCi or μCi amounts of radioactivity)? Not much, for the obvious reason that direct studies have not been undertaken. Accordingly, guidelines for exposure levels are set using extrapolations—either by extrapolating down from population statistics obtained following accidents or disasters (the Chernobyl meltdown, atomic bombings) or by extrapolating up from numbers obtained from animal experiments. Each form of extrapolation is subject to caveats, and given that predictions based on such extrapolations cannot be perfect, most health and safety personnel aim for radiation exposure levels said to be ALARA or "as low as reasonably achievable." Further discussion of exposure limits and the statistics on which they are based may be found in B. Shleien's health physics text (Shleien, 1987). Limiting exposure to radiation can be accomplished by adjusting several parameters of the exposure: the duration of exposure, distance from the source, and the density of the material (air, water, shielding) between the individual and the source.

Time is of the essence

When designing any experiment using radioactivity, every effort should be made to limit the time spent directly handling the vials or tubes containing the radioactive material. Speed should be encouraged in all manipulations, though not to the point of recklessness! Have everything needed for the experiment ready at hand before the radioactivity is introduced into the work area.

Distance helps to determine dose

When possible, experiments involving radioactivity should be performed in an area separate from the rest of the lab. Many institutions require that such work be performed in a designated "hot lab"; however, if many people in the laboratory routinely use radioisotopes, it is less than feasible to move them all into what is usually a smaller space. No matter where an individual is working, it is his or her responsibility to monitor the work area and ensure his or her own safety and the safety of those working nearby by using adequate shielding. Obviously, when handling the radioactive samples, it is necessary to work rapidly behind any required shielding. To protect bystanders, remember that the intensity of radiation from a source (moving through air) falls off in proportion to the square of the distance. Thus, if standing 1 foot away from a source for 5 min would result in an exposure of 45 units, standing 3 feet away for the same amount of time would result in an exposure $(1/3)^2$ of 45 units,

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or 5 units. This factor is also relevant when considering the storage of large amounts of radioactivity, particularly ^{125}I or ^{32}P, as no amount of shielding can completely eliminate radiation.

Shielding is the key to safety

As mentioned above, the energy of the particle(s) released during the decay of an isotope determines what, if any, type of shielding is appropriate. β particles released during the decay of ^{14}C and ^{35}S possess roughly ten times the energy of those released when ^{3}H decays. All three β particles are of relatively low energy, do not travel very far in air, and cannot penetrate solid surfaces. No barriers are necessary for shielding against this type of β radiation. The major health threat from these isotopes occurs through their accidental ingestion, inhalation, or injection.

β particles released during the decay of ^{32}P have 10-fold higher energy than those released from ^{14}C and pose a significant threat to workers. (One reported hazard is the potential for induction of cataracts in the unshielded eye.) The fact that these high-energy beta particles can potentially generate significant amounts of Bremsstrahlung radiation is the reason that low-density materials are used as the primary layer of shielding for ^{32}P β radiation. Water, glass, and plastic are suitable low-density materials (as opposed to lead). Obviously water is unsuitable as a shielding layer for work on the bench, although it does a reasonable job when samples are incubating in a water bath. Shields made from a thickness of glass sufficient to stop these particles would be extremely heavy and cumbersome (as well as dangerous if dropped). Fortunately, plastic or acrylic materials—variously called Plexiglas, Perspex, or Lucite—are available for shielding against ^{32}P β radiation. Shields as well as storage boxes constructed of various thicknesses of Plexiglas are necessary equipment in laboratories where ^{32}P is used. When mCi amounts of ^{32}P are used at one time it is necessary to also block the Bremsstrahlung radiation by adding a layer of high-density material (such as 4 to 6 mm lead) to the outside of the Plexiglas shield (covering the side farthest from the radioactive source).

γ rays released during the decay of ^{125}I have much higher penetrance than the β particles from ^{32}P decay; this radiation must be stopped by very-high-density material, such as lead. Lead foil of varying thicknesses (2 to 6 mm) can be purchased in rolls and can be cut and molded to cover any container, or taped to a Plexiglas shield (used in this instance for support). Obviously this latter arrangement has the disadvantage that it is impossible to see what one is doing through the shield. For routine shielding of manipulations involving ^{125}I, it is useful to purchase a lead-impregnated Plexiglas shield that is transparent, albeit inevitably very heavy (as well as relatively expensive).

Although it seems logical that the use of more radioactivity necessitates the use of thicker layers of shielding, it is also true that no shielding material is capable of completely stopping all radiation. When deciding how thick is "thick enough," consult the half-value layer measurement for each type of shielding material. This number gives the thickness of a given material necessary to stop half the radiation from a source; Table A.2B.2 lists half-value layer measurements for the β particles released from isotopes commonly used in the biology laboratory. In general, 1 to 2 cm of Plexiglas and/or 0.02 mm of lead are sufficient to shield the amounts of radioactivity used in our experiments.

GENERAL PRECAUTIONS

Before going on to a discussion of specific precautions to be taken with individual isotopes, a short list of general precautions to be taken with all isotopes seems pertinent:

1. Know the rules. Be sure that each individual is authorized to use each particular isotope and uses it in an authorized work area.

2. Don the appropriate apparel. Whenever working at the lab bench, it is good safety practice to wear a labcoat for protection. Disposable paper/synthetic coats of various styles are commercially available: at $4 each these may be conveniently thrown out if contaminated with radioactivity during an experiment, rather than held for decay as might be preferable with cloth coats costing about $30 each. As an alternative, disposable sleeves can be purchased and worn over those of the usual cloth coat. Other necessary accessories include radiation safety badges, gloves, and protective eyewear. It's handy to wear two pairs of gloves at once when using radioactivity; when the outer pair becomes contaminated, it is possible to strip it off and continue working without interruption.

3. Protect the work area as well as the workers. Lab benches and the bases of any shields used should be covered with some sort of disposable, preferably absorbent, paper sheet. Underpads or diapers (the kind normally

Table A.2B.2 Shielding Radioactive Emission[a]

β emitters

Energy (MeV)	Mass (mg)/cm² to reduce intensity by 50%	Thickness (mm) to reduce intensity by 50%			
		Water	Glass	Lead	Plexiglas
0.1	1.3	0.013	0.005	0.0011	0.0125
1.0	48	0.48	0.192	0.042	0.38
2.0	130	1.3	0.52	0.115	1.1
5.0	400	4.0	1.6	0.35	4.2

γ emitters

Energy (MeV)	Thickness of material (cm) to attenuate a broad beam of γ-rays by a factor of 10			
	Water	Aluminum	Iron	Lead
0.5	54.6	20.3	6.1	1.8
1.0	70.0	24.4	8.2	3.8
2.0	76.0	32.0	11.0	5.9
3.0	89.0	37.0	12.0	6.4

[a]From Dawson et al. (1986). Reprinted with permission.

used in hospitals) are convenient for this purpose.

4. Use appropriate designated equipment. It is very convenient, where use justifies the expense, to have a few adjustable pipettors dedicated for use with each particular isotope. Likewise, it is good practice to use only certain labeled centrifuges and microcentrifuge rotors for radioactive samples so that all the lab's rotors do not become contaminated. Although such equipment should be cleaned after each use, complete decontamination is often not possible. A few pipettors or a single microcentrifuge can easily be stored (and used) behind appropriate shielding. Actually, contamination of the insides and tip ends of pipettors can be greatly reduced by using tips supplied with internal aerosol barriers; these have recently become very popular for setting up PCR reactions and are available to fit a variety of pipettors. To prevent contamination of the outside of the pipettor's barrel, simply wrap the hand-grip in Parafilm, which can be discarded later. Several manufacturers sell disposable paper inserts for their microcentrifuges that protect the wall of the rotor chamber from contamination that might spin off the outside of sample tubes. Trying to fashion homemade liners of this sort is not recommended, as we have had disastrous experiences using laminated adhesive paper that "unstuck" during microcentrifuge spins. These liners would get caught by the rotor, shattering sample tubes and creating an even bigger mess!

5. Know where to dispose of radioactive waste, both liquid and solid. Most institutions require that radioactive waste be segregated by isotope. This is done not only so that appropriate shielding can be placed around waste containers, but so that some waste can be allowed to decay prior to disposal through normal trash channels. With a decreasing number of radioactive waste disposal facilities able or willing to accept radioactive waste for burial (and a concomitant increase in dumping charges from those that still do) this practice of on-site decay can save an institution thousands of dollars a year in disposal charges.

6. Label your label! It is only common courtesy (as well as common sense) to alert coworkers to the existence of anything and everything radioactive that is left where they may come in contact with it! A simple piece of tape affixed to the sample box—with the investigator's name, the amount and type of isotope, and the date written on it—should do the trick. Yellow hazard tape printed with the international symbol for radioactivity is commercially available in a variety of widths.

7. Monitor radioactivity early and often. Portable radiation detection monitors are essential equipment for every laboratory using radioactivity. No matter how much or how little radioactivity is being used, the investigator

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should keep a hand-held monitor nearby—and it should be on! Turn it on before touching any radioactivity to avoid contaminating the monitor's switch. Use a monitor with the appropriate detection capacity (β for ^{35}S and ^{32}P; γ for ^{125}I) before, during, and after all procedures. The more frequently fingers and relevant equipment are monitored, the more quickly a spill or glove contamination will be detected. Such timely detection will keep both the potential mess and the cleanup time to a minimum. Because low-energy β emitters such as ^{3}H cannot be detected using such monitors, wipe tests of the bench and equipment used are necessary to ensure that contamination of the work area did not occur.

SPECIFIC PRECAUTIONS

The following sections describe precautions to be taken with individual isotopes in specific forms. Although the sections dealing with ^{35}S- or ^{32}P-labeling of proteins in intact cells are presented in terms of mammalian cells, most of the instructions are also pertinent (with minimal and obvious modifications) to the labeling of proteins in other cells (e.g., bacterial, insect).

Working with ^{35}S

Using ^{35}S to label cellular proteins and proteins translated in vitro

As discussed above, the β radiation generated during ^{35}S decay is not strong enough to make barrier forms of shielding necessary. The risk associated with ^{35}S comes primarily through its ingestion and subsequent concentration in various target organs, particularly the testes. Although willful ingestion of ^{35}S seems unlikely, accidental or unknowing ingestion may be more common. As reported several years ago (Meisenhelder and Hunter, 1988), ^{35}S-labeled methionine and cysteine, which are routinely used to label proteins in intact cells and by in vitro translation, break down chemically to generate a volatile radioactive component. The breakdown occurs independent of cellular metabolism. Thus the radioactive component is generated to the same extent in stock vials as in cell culture dishes. The process seems to be promoted by freezing and thawing ^{35}S-labeled materials. The exact identity of this component is not known, although it is probably SO_2 or CH_3SH. What is known is that it dissolves readily in water and is absorbed by activated charcoal or copper.

The amount of this volatile radioactive component released, despite stabilizers added by the manufacturers, is about 1/8000 of the total radioactivity present. The amount of this radioactivity that a scientist is likely to inhale while using these compounds is presumably even smaller. Nevertheless, such a component can potentially contaminate a wide area because of its volatility, and also tends to concentrate in target organs. Thus, it is advisable to thaw vials of ^{35}S-labeled amino acids in a controlled area such as a hood equipped with a charcoal filter. This charcoal filter will become quite contaminated and should be changed every few months. If such an area is not available, the stock vial should be thawed using a needle attached to a charcoal-packed syringe to vent and trap the volatile compound.

Anyone who has ever added ^{35}S-labeled amino acids to dishes of cells for even short periods knows that the incubator(s) used for such labelings quickly become highly contaminated with ^{35}S. Such contamination is not limited to the dish itself, nor to the shelf on which the dish was placed. Rather, the radioactive component's solubility in water allows it to circulate throughout the moist atmosphere of the incubator and contaminate all the inside surfaces of the incubator. For this reason, in laboratories where such metabolic labelings are routine, it is highly convenient to designate one incubator to be used solely for working with ^{35}S-labeled samples. Such an incubator can be fitted with a large honeycomb-style filter, the size of an incubator shelf, made of pressed, activated charcoal. These filters are available from local air-quality-control companies. Such a filter will quickly become quite contaminated with radioactivity and should therefore be monitored and changed as necessary (usually about every three months if the incubator is used several times a week). The water used to humidify the incubator will also become quite "hot" (contaminated with radioactivity); keeping the water in a shallow glass pan on the bottom of the incubator makes it easy to change it after every use, thus preventing contamination from accumulating. Even with the charcoal filter and water as absorbents, the shelves, fan, and inner glass door of the incubator will become contaminated, as will the tray on which the cells are carried and incubated. Routine wipe tests and cleaning when necessary will help to minimize potential spread of this contamination.

If such labelings are done infrequently or there is no "spare" incubator, dishes of cells can be placed in a box during incubation. This box should be made of plastic, which is generally

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more easily decontaminated than metal. Along with the dishes of cells, a small sachet made of activated charcoal wrapped loosely in tissue (Kimwipes work well) should be placed in the box. If the box is sealed, it will obviously need to be gassed with the correct mixture of CO_2; otherwise small holes can be incorporated into the box design to allow equilibration with the incubator's atmosphere. In either case, the incubator used for the labeling should be carefully monitored for radioactivity after each experiment.

Working with ^{32}P

μCi amounts of ^{32}P

The amount of ^{32}P-labeled nucleotide used to label nucleic acid probes for northern or Southern blotting is typically under 250 μCi, and the amount of [γ-^{32}P]ATP used for in vitro phosphorylation of proteins does not usually exceed 50 μCi for a single kinase reaction (or several hundred μCi per experiment). However, handling even these small amounts, given the time spent on such experiments, can result in an unacceptable level of exposure if proper shielding is not employed. With no intervening shielding, the dose rate 1 cm away from 1 mCi ^{32}P is 200,000 mrads/hr; the local dose rate to basal cells resulting from a skin contamination of 1 μCi/cm^2 is 9200 mrads/hr (Shleien, 1987). Such a skin contamination could be easily attained though careless pipetting and the resultant creation of an aerosol of radioactive microdroplets, because the concentration of a typical stock solution of labeled nucleotide may be 10 μCi/μl.

For proper protection during this sort of experiment, besides the usual personal attire (glasses, coat, safety badges, and gloves) it is necessary to use some form of Plexiglas screen between the body and the samples (see Fig. A.2B.1, panel A). Check the level of radiation coming through the outside of the shield with a portable monitor to be sure the thickness of the Plexiglas is adequate. Hands can be shielded from some exposure by placing the sample tubes in a solid Plexiglas rack, which is also useful for transporting samples from the bench to a centrifuge or water bath (see Fig. A.2B.1, panel B).

Experiments of these types often include an incubation step performed at a specific temperature, usually in a water bath. Although the water surrounding the tubes or hybridization bags will effectively stop β radiation, shielding should be added over the top of the tubes (where there is no water)—e.g., a simple flat piece of Plexiglas. If the frequency of usage justifies the expense, an entire lid for the water bath can be constructed from Plexiglas. When hybridization reactions are performed in bags, care should be taken to monitor (and shield) the apparatus used to heat-seal the bags.

The waste generated during the experiments should also be shielded. It is convenient to have a temporary waste container right on the bench. Discard pipette tips and other solid waste into a beaker lined with a plastic bag and placed behind the shield. This bag can then be emptied into the appropriate shielded laboratory waste container when the experiment is done. Liquid waste can be pipetted into a disposable tube set in a stable rack behind the shield (see Fig. A.2B.1, panel C).

When radiolabeled probes or proteins must be gel-purified, it may be necessary to shield the gel apparatus during electrophoresis if the samples are particularly hot. Be advised that the electrophoresis buffer is likely to become very radioactive if the unincorporated label is allowed to run off the bottom of the gel; check with a radiation safety officer for instructions on how to dispose of such buffer. It is also prudent to check the gel plates with a portable detection monitor after the electrophoresis is completed, because they sometimes become contaminated as well.

mCi amounts of ^{32}P

In order to study protein phosphorylation in intact mammalian cells, cells in tissue culture dishes are incubated in phosphate-free medium with ^{32}P-labeled orthophosphate for a period of several hours or overnight to label the proteins. The amount of ^{32}P used in such labelings can be substantial. Cells are normally incubated in 1 to 2 mCi of ^{32}P/ml labeling medium; for each 6-cm dish of cells, 2.5 to 5 mCi ^{32}P may be used. When this figure is multiplied by the number of dishes necessary per sample, and the number of different samples in each experiment, it is clear that the amount of ^{32}P used in one experiment can easily reach 25 mCi or more. Because so much radioactivity is used in the initial labeling phase of such experiments, it is necessary for a researcher to take extra precautions in order to adequately shield him or herself and coworkers.

When adding label to dishes of cells, it is important to work as rapidly as possible. An important contribution to the speed of these manipulations is to have everything that will be needed at hand before even introducing the

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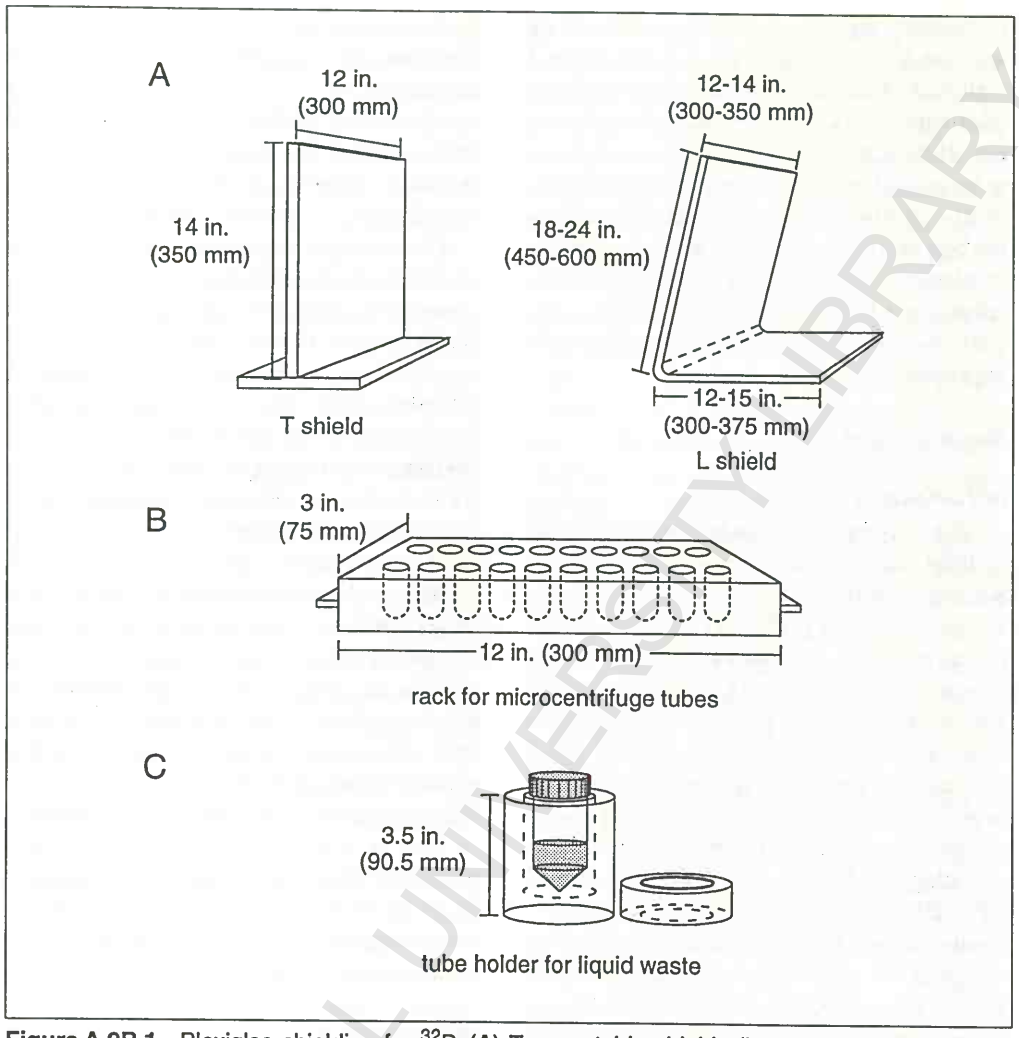

Figure A.2B.1 Plexiglas shielding for ^{32}P. (**A**) Two portable shields (L and T design) made of 0.5-in. Plexiglas. Either can be used to directly shield the scientist from the radioactivity he or she is using. Turned on its side, the L-shaped shield can be used to construct two sides of a cage around a temporary work area, providing shielding for other workers directly across or to the sides of the person working with ^{32}P. (**B**) Tube rack for samples in microcentrifuge tubes. (**C**) Tube holder for liquid waste collection.

label into the work area. Prepare the work area, arranging shielding and covering the bench with diapers, in advance. Set out all necessary items, including any pipettors and tips needed, a portable detection monitor, extra gloves, and a cell house (see Fig. A.2B.2, panel A).

Work involving this much radioactivity should be done behind a Plexiglas shield at least ¾ in. (2 cm) thick; the addition of a layer of lead to the outside lower section of this shield to stop Bremsstrahlung radiation is also needed. If one shield can be dedicated to this purpose at a specific location, a sheet of lead several centimeters thick can be permanently screwed to the Plexiglas (as shown in Fig. A.2B.2, panel B); however, this lead makes the shield extremely heavy and therefore less than portable. If space constraints do not permit the existence of such a permanent labeling station, a layer or two of lead foil can be taped temporarily to the outside of the Plexiglas shield.

Again, each worker should take care to shield not only him or herself, but bystanders on all sides. Handling of label should be done away from the central laboratory if possible to take maximum advantage of distance as an additional form of shielding. It is also advisable not to perform such experiments in a tissue culture room or any other room that is designed for a purpose vital to the whole laboratory. An accident involving this much ^{32}P would seriously inconvenience future work in the area, if not make it altogether uninhabitable! If care is taken to minimize the amount of time the dish

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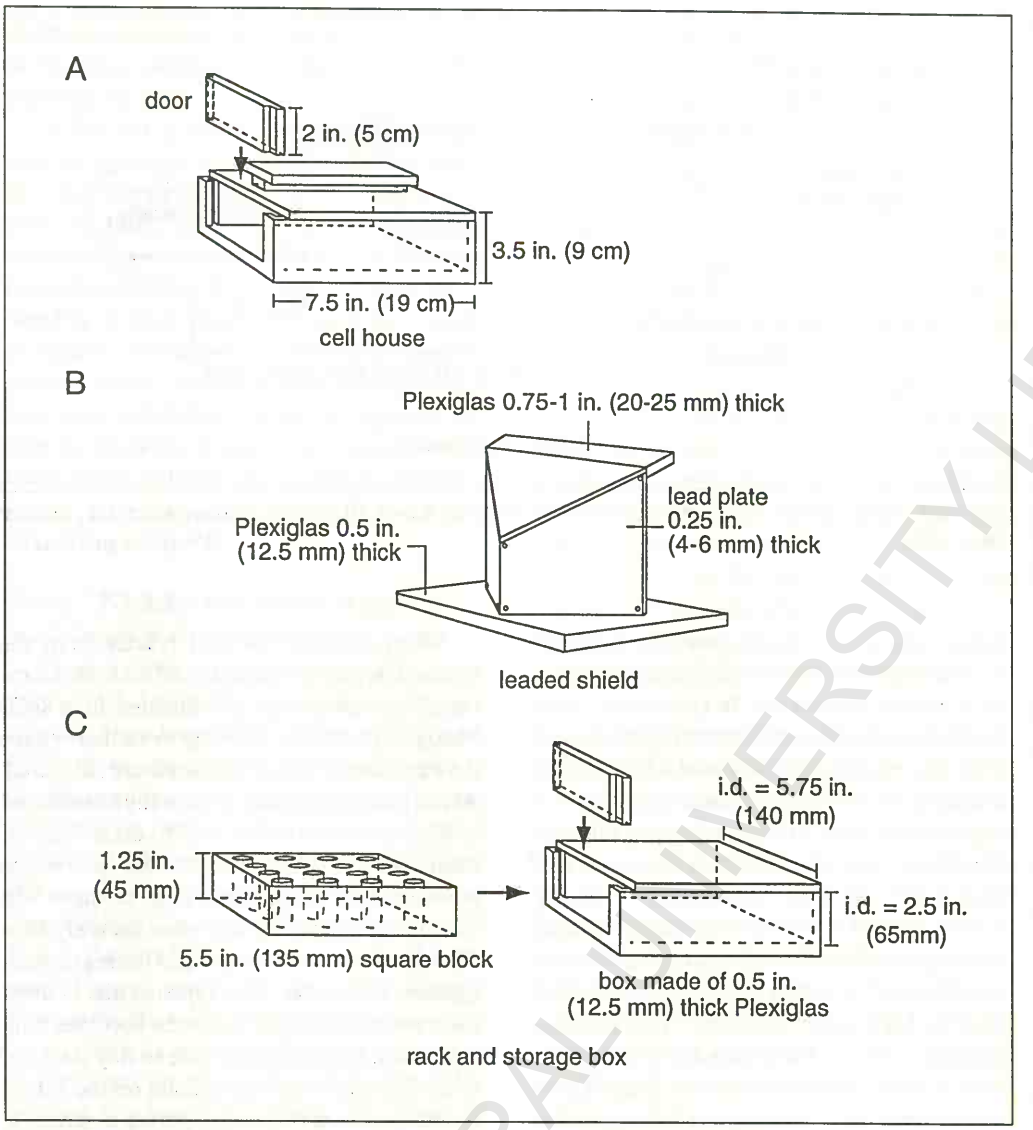

Figure A.2B.2 (**A**) Box for cell incubation (a "cell house"). (**B**) Stationary leaded shield. (**C**) Sample storage rack and box made of 0.5-in. Plexiglas. Abbreviations: i.d., interior dimension.

of cells is open when adding the label, use of a controlled air hood to prevent fungal or bacterial contamination of the cells should not be necessary.

Once the label has been added to the dishes of cells, they will also need to be shielded for transport to and from the incubator and other work areas. Plexiglas boxes that are open at one end (for insertion of the dishes) and have a handle on top (for safe carrying) make ideal "cell houses" (see Figure A.2B.2, panel A). A Plexiglas door that slides into grooves at the open end is important to prevent dishes from sliding out if the box is tilted at all during transport. If this door is only two-thirds the height of the house wall, the open slot thus created will allow equilibration of the CO_2 level within the house with that in the incubator. Obviously, this slot will also allow a substantial stream of radiation to pass out of the cell house, so the house should be carried and placed in the incubator with its door facing away from the worker (and others)!

Following incubation with label and any treatments or other experimental manipulations, the cells are usually lysed in some type of detergent buffer. It is probably during this lysis procedure that a worker's hands will receive their greatest exposure to radiation, because it is necessary to directly handle the dishes over a period of several minutes. It is therefore very important to streamline this procedure and use any shielding whenever possible. If the cell lysates must be made at 4°C, as

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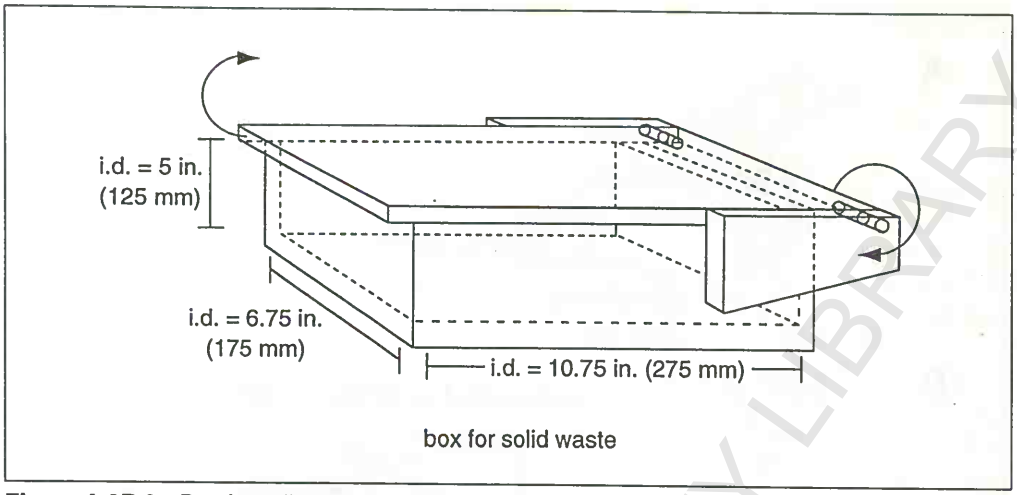

Figure A.2B.3 Box for solid waste collection made of 0.5-in. Plexiglas. Abbreviation: i.d., interior dimension.

required by most protocols, working on a bench in a cold room is preferable to placing the dishes on a slippery bed of ice. In either case, make the lysate using the same sort of shielding (with lead if necessary) that was used when initially adding the label. Pipet the labeling medium and any solution used to rinse unincorporated radioactivity from the cells into a small tube held in a solid Plexiglas holder (shown in Fig. A.2B.1, panel C). The contents of this tube can later be poured into the appropriate liquid waste receptacle. If possible, it is a good practice to keep this high-specific-activity ^{32}P liquid waste separate from the lower-activity waste generated in other procedures so that it can be removed from the laboratory as soon as possible following the experiment. If it is necessary to store it in the laboratory for any time, the shielding for the waste container should also include a layer of lead.

The solid waste generated in the lysis part of these experiments (pipet tips, disposable pipets, cell scrapers, and dishes) is very hot and should be placed immediately into some sort of shielded container to avoid further exposure of the hands. A Plexiglas box similar in design to that in Figure A.2B.3 is convenient; placed to the side of the shield and lined with a plastic bag, it will safely hold all radioactive waste during the experiment and is light enough to be easily carried to the main laboratory waste container where the plastic bag (and its contents) can be dumped after the experiment is done. If the lid of the box protrudes an inch or so over the front wall, it can be lifted using the back of a hand, thus decreasing the possibility of contaminating it with hot gloves.

When scraping the cell lysates from the dishes, it is good practice to add them to microcentrifuge tubes that are shielded in a solid Plexiglas rack; this will help to further reduce the exposure to which the hands are subjected. At this point, the lysates are usually centrifuged at high speed (>10,000 × *g*) to clear them of unsolubilized cell material. Use screw-cap tubes for this clarification step, as these will contain the labeled lysate more securely than flip-top tubes, which may open during centrifugation. No matter what type of tube is used, the rotor of the centrifuge often becomes contaminated, most probably due to tiny drops of lysate (aerosol) initially present on the rim of the tubes that are spun off during centrifugation. Monitor the rotor and wipe it out after each use.

The amount of ^{32}P taken up by cells during the incubation period varies considerably, depending on the growth state of the culture as well as on the cell type and its sensitivity to radiation. This makes it difficult to predict the percentage of the radioactivity initially added to the cells that is incorporated into the cell lysate; however, this figure probably does not exceed 10%. Thus, the amount of radioactivity being handled decreases dramatically after lysis, making effective shielding much simpler. However, at least ten times more radioactivity than is usual in other sorts of experiments is still involved! It is easy to determine if the shielding is adequate—just use both β and γ portable monitors to measure the radiation coming through it. Again, be sure to check that people working nearby (including those across the bench) are also adequately shielded. It is

sometimes necessary to construct a sort of cage of Plexiglas shields around the ice bucket that contains the lysates.

At the end of the day or the experiment, it may be necessary to store radioactive samples; in some experiments, it may be desirable to save the cell lysates. These very hot samples are best stored in tubes placed in solid Plexiglas racks that can then be put into Plexiglas boxes (see Fig. A.2B.2, panel C). Such boxes may be of similar construction to the cell houses described above; however, they should have a door that completely covers the opening. Be sure to check the γ radiation coming through these layers and add lead outside if necessary.

Working with ^{33}P

Using ^{33}P-labeled nucleotides to label nucleic acid probes or proteins

Several of the major companies that manufacture radiolabeled biological molecules have recently introduced nucleotides labeled with ^{33}P (both α- and γ-labeled forms). ^{33}P offers a clear advantage over ^{32}P with respect to ease of handling, because the energy of the β particles it releases lies between that of ^{35}S and ^{32}P and thus its use does not require as many layers of Plexiglas and lead shielding as for ^{32}P. In fact, the β radiation emitted can barely penetrate through gloves and the surface layer of skin, so the hazard associated with exposure to even millicurie amounts of ^{33}P is thought to be insignificant (as reported in the DuPont NEN product brochure). Gel bands visualized on autoradiographs of ^{33}P-labeled compounds are sharper than bands labeled with ^{32}P because the lower-energy β radiation does not have the scatter associated with that from ^{32}P. The half-life of ^{33}P is also longer (25 days compared to 14 days for ^{32}P). Despite its higher cost, these features have led many researchers to choose ^{33}P-labeled nucleotides for use in experiments such as band/gel shift assays where discrimination of closely-spaced gel bands is important.

The best way to determine what degree of shielding is needed when using ^{33}P is to monitor the source using a portable β monitor and add layers of Plexiglas as necessary.

Working with ^{125}I

Using ^{125}I to detect immune complexes (Western blots)

^{125}I that is covalently attached to a molecule such as staphylococcal protein A is not volatile and therefore is much less hazardous than the unbound or free form. Most institutions do not insist that work with bound ^{125}I be performed in a hood, but shielding of the γ radiation is still necessary. Lead is a good high-density material for stopping these γ rays; its drawbacks are its weight and opacity. Commercially available shields for ^{125}I are made of lead-impregnated Plexiglas—though heavy, these are at least see-through. Alternatively, a piece of lead foil may be taped to a structural support, although this arrangement does not provide shielding for the head as a worker peers over the lead!

Incubations of the membrane or blot with the [^{125}I] protein A solution and subsequent washes are usually done on a shaker. For shielding during these steps, a piece of lead foil may simply be wrapped around the container. Solutions of ^{125}I can be conveniently stored for repeated use in a rack placed in a lead box.

Using ^{125}I to label proteins or peptides in vitro

Any experiments that call for the use of free, unbound ^{125}I should be done behind a shield in a hood that contains a charcoal filter to absorb the volatile iodine. Most institutions require that such experiments be done in a special hot lab to which access is limited. Ingested or inhaled iodine is concentrated in the thyroid; a portable γ monitor should therefore be used to scan the neck and throat before beginning and after completing each experiment. Similar scans should routinely be performed on all members of any laboratory in which unbound iodine is used.

DEALING WITH ACCIDENTS

Despite the best intentions and utmost caution, accidents happen! Accidents involving spills of radioactivity are particularly insidious because they can be virtually undetectable yet pose a significant threat to laboratory workers. For this reason it is best to foster a community spirit in any laboratory where radioisotopes are routinely used—a sense of cooperativity that extends from shielding each other properly to helping each other clean up when such accidents occur.

The specific measures to be taken following an accident involving radioactivity naturally depend on the type and amount of the isotope involved, the chemical or biological hazards of the material it is associated with, and the physical parameters of the spill (i.e., where and onto what the isotope was "misplaced"). However, following any accident there are several immediate steps that should be taken:

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1. Alert coworkers as well as Radiation Safety personnel to the fact that there has been an accident. This will give them the opportunity to shield themselves if necessary—and to help clean up as well!

2. Restrict access to and away from the site of the accident to ensure that any uncontained radioactive material is not spread around the laboratory. When leaving the site be sure to monitor the bottoms of the shoes as well as the rest of the body.

3. Take care of all contaminated personnel first, evacuating others if necessary. If anyone's skin is contaminated, first use a portable monitor to identify specific areas of contamination. Then wipe these areas with a damp tissue to remove as much surface radioactivity as possible. Try to scrub only small areas at a time to keep the contamination localized. If the contamination is not easily removed with paper tissues, try a sponge or an abrasive pad, but be careful not to break the skin! Sometimes soaking is required: do this only after all easily removed contamination is gone and keep the soaked area to a minimum. Contaminated strands of hair can be washed (or perhaps a new hairstyle may be in order).

4. When attempting to clean any contaminated equipment, floors, benches, etc., begin by soaking up any visible radioactive liquid with an absorbent material. Use a small amount of soap and water to clean the contaminated area, keeping the area wiped each time to a minimum to avoid smearing the contamination over an even greater surface. Many surfaces prove resistant to even Herculean cleaning efforts; in these instances the best that can be done is to remove all contamination possible and then shield whatever is left until the radioactivity decays sufficiently for safety.

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Contributed by Jill Meisenhelder and Kentaro Semba
The Salk Institute
La Jolla, California

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Centrifuges and Rotors

Centrifugation runs described in this book usually specify a relative centrifugal force (RCF; measured in $\times g$), corresponding to a speed (in rpm) for a particular centrifuge and rotor model. As available equipment will vary from laboratory to laboratory, the investigator must be able to adapt these specifications to other centrifuges and rotors.

The relationship between RCF and speed (rpm) is determined by the following equation:

$$RCF = 1.12r\,(rpm/1000)^2$$

where r is the rotating radius between the particle being centrifuged and the axis of rotation. In most cases, an accurate conversion from speed to relative centrifugal force (or vice versa) can be obtained using the maximum value of r—or r_{max}—equal to the distance between the axis of rotation and the bottom of the centrifuge tube as it sits in the well or bucket of the rotor.

Table A.2C.1 provides r_{max} values for commonly used rotors manufactured by Du Pont (Sorvall), Beckman, Fisher, and IEC. There are situations (e.g., where an adapter is used to fit a smaller tube into a larger rotor well) where r_{max} will not accurately represent the effective rotating radius. In such cases, the manual for the rotor should be consulted to obtain the appropriate value of r.

As an alternative to use of the above equation, the nomograms in Figures A.2C.1 and A.2C.2 make it possible to determine the RCF where speed and r_{max} are known, or the speed where RCF and r_{max} are known. This is done by aligning a ruler across the two known values and reading the unknown value at the point where the ruler crosses the remaining column. Figure A.2C.1 should be used for centrifuge runs <21,000 rpm, while Figure A.2C.2 should be used for faster spins.

NOTE: In this manual, for spins involving microcentrifuges built to the Eppendorf standard, a shortened style of reference including only the speed (in rpm) is used. All of these instruments have approximately the same rotating radius; hence the same speed will yield the same RCF value from machine to machine. Microcentrifuge spins may also be described as at "top speed" or "maximum speed," meaning 12,000 to 14,000 rpm, which is the maximum speed for all Eppendorf-type microcentrifuges.

CAUTION: Do not exceed maximum rotor speed! For Beckman ultracentrifuges, the maximum speed for each rotor is denoted by its name, e.g., the maximum speed of the Beckman VTi 80 rotor is 80,000 rpm. This speed refers only to centrifugation of solutions below a particular allowed density, which differs among rotors (see user manual). For centrifugation of high-density solutions, rotor maximum speed can be determined as: reduced rpm = $rpm_{max}(A/B)^{1/2}$, where A = allowed density and B = density of solution. A = 1.7 g/ml for several vertical rotors (including VTi 80 and VTi 50), and 1.2 g/ml for several swinging-bucket rotors (including SW 55 Ti, 28, 28.1, 40 Ti, and 50.1). For gradients using heavy salts such as CsCl, particularly at low temperatures, maximum rpm should be reduced to prevent precipitation (see user manual).

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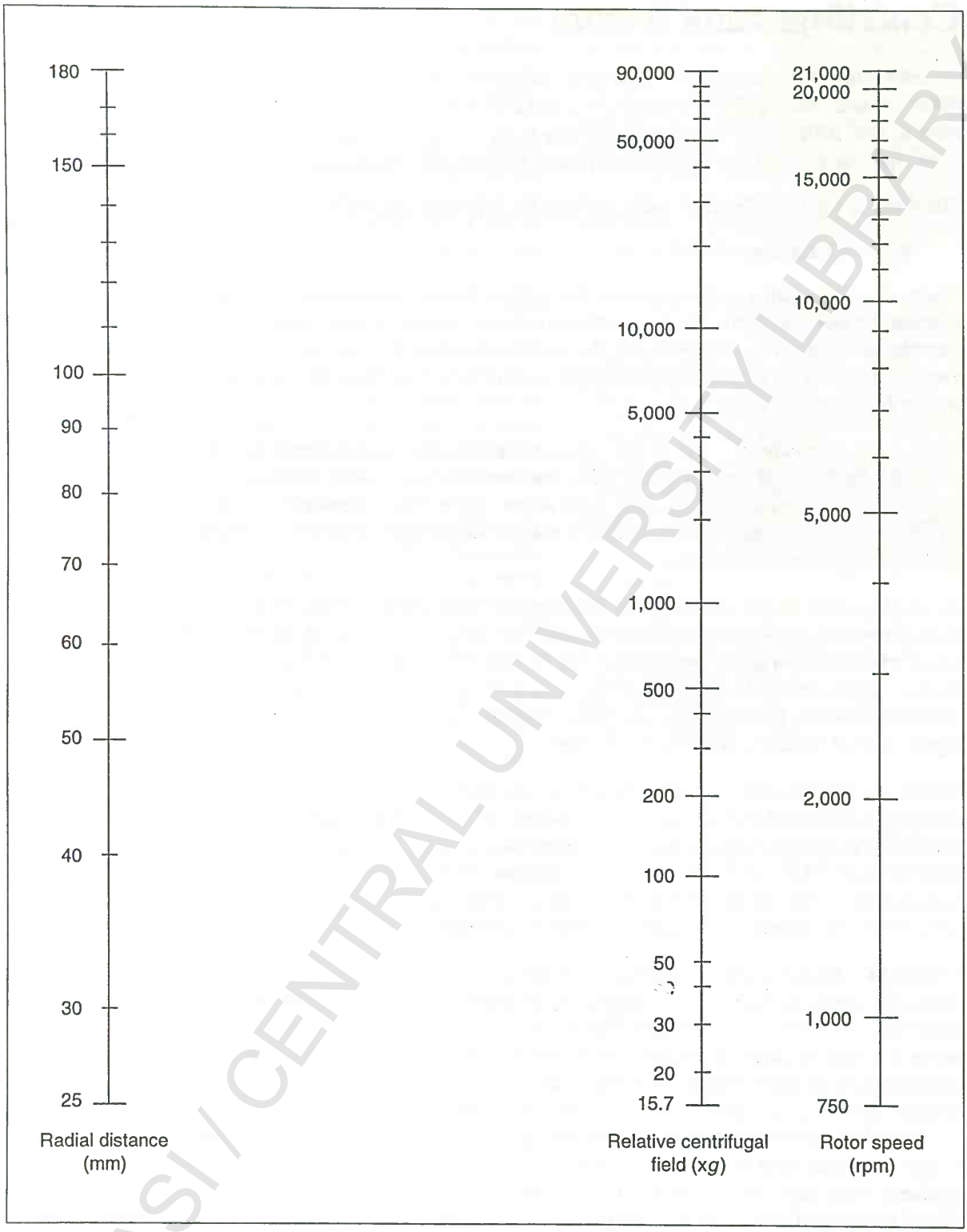

Figure A.2C.1 Nomogram for conversion of relative centrifugal force to rotor speed in low-speed centrifuge runs. For faster centrifugations, use Figure A.2C.2. A more precise conversion can be obtained using the equation at the beginning of this appendix. See Table A.2C.1 for rotating radii of commonly used rotors.

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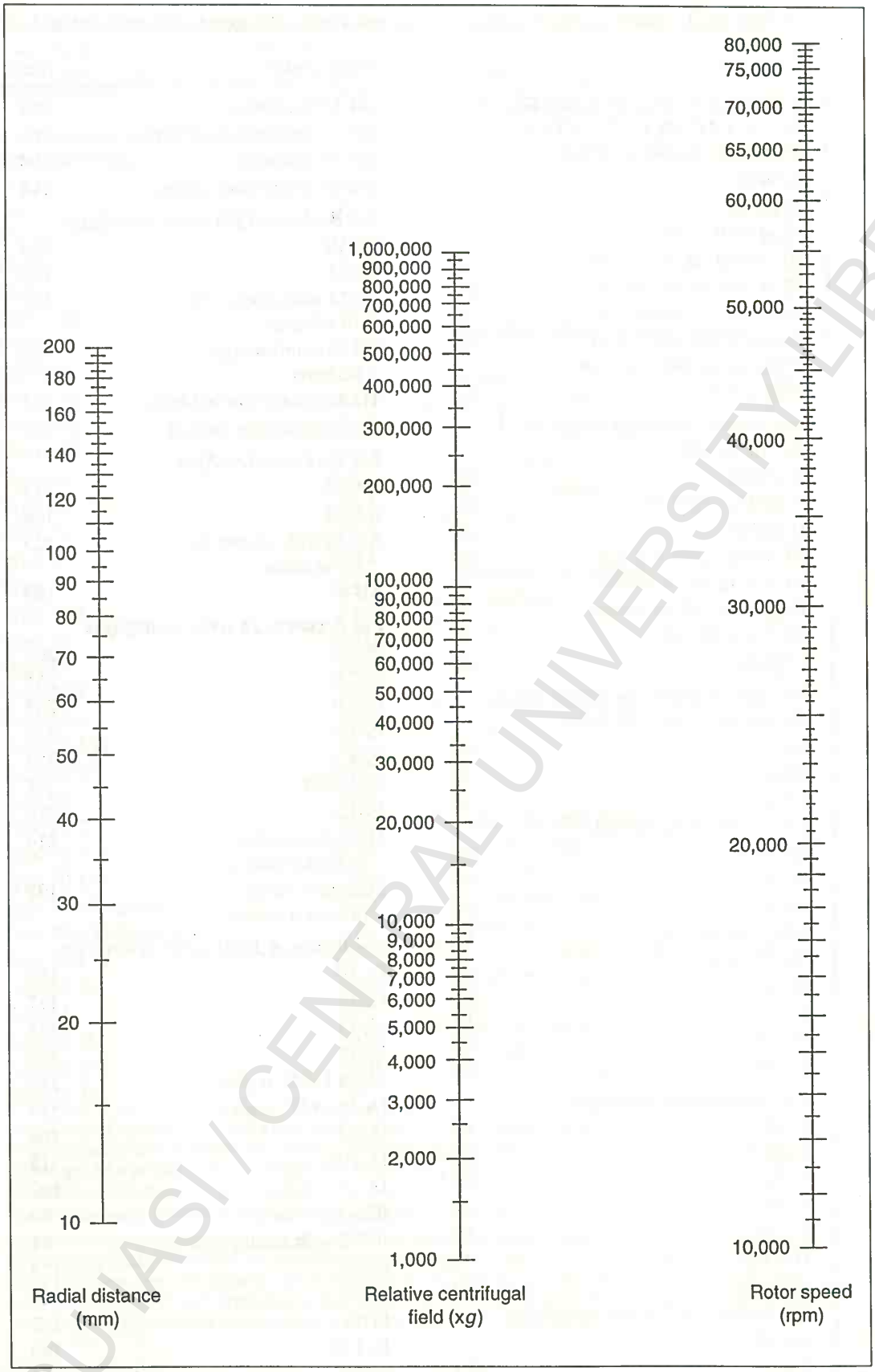

Figure A.2C.2 Nomogram for conversion of relative centrifugal force to rotor speed in high-speed centrifuge runs. For slower centrifugations, use Figure A.2C.1. A more precise conversion can be obtained using the equation at the beginning of this appendix. See Table A.2C.1 for rotating radii of commonly used rotors.

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Table A.2C.1 Maximum Rotating Radii for Common Rotors, Grouped by Centrifuge Model

Rotor model[a]	r_{max} (mm)
For Sorvall centrifuge models GLC-1, GLC-2, GLC-2B, GLC-3, GLC-4, RT-6000B, T-6000, T-6000B	
A/S400	140
H-1000B	186
HL-4 with 50-ml bucket	180
HL-4 with 100-ml bucket	204
HL-4 with Omni-Carrier	163
M and A-384 (inner row)	91
M and A-384 (outer row)	121
SP/X and A-500 (inner row)	82
SP/X and A-500 (outer row)	123
For Sorvall centrifuge models RC-3, RC-3B, RC-3C	
H-2000B	261
H-4000 and HG-4L	230
H-6000A	260
HL-8 with Omni-Carrier	221
HL-8 with 50-ml bucket	238
HL-8 with 100-ml bucket	247
HL-2 and HL-2B	166
LA/S400	140
For Sorvall centrifuge models RC-2, RC-2B, RC-5, RC-5B, RC-5C	
GSA	145
GS-3	151
HB-4	147
HS-4 with 250-ml bucket	172
SA-600	129
SE-12	93
SH-80	101
SM-24 (inner row)	91
SM-24 (outer row)	110
SS-34	107
SV-80	101
SV-288	90
TZ-28	95
For Sorvall ultracentrifuges	
T-865	91
T-865.1	87.1
T-875	87.1
T-880	84.7
T-1270	82
TFT-80.2	65.5
TFT-80.4	60.1
For Beckman GP series centrifuges	
GA-10	123
GA-24	123
GA-24 with adapter for 10-ml tubes	108
GH-3.7 (buckets)	204
GH-3.7 (microplate carrier)	168
GH-3.8 (buckets)	204
GH-3.8 (microplate carrier)	168
For Beckman TJ-6 series centrifuges	
TA-10	123
TA-24	108
TA-24 with adapter for 10-ml tubes	123
TH-4 (stainless-steel buckets)	186
TH-4 (100-ml tube holders)	201
TH-4 (microplate carrier)	165
For Beckman AccuSpin	
AA-10	123
AA-24	108
AA-24 with adapter for 10-ml tubes	123
AH-4	163
For Beckman J6 series centrifuges	
JR-3.2	206
JS-2.9	265
JS-3.0	254
JS-4.0	226
JS-4.2	254
JS-4.2SM	248
JS-5.2	226
Microplate carrier (6-bucket rotors)	214
Microplate carrier (4-bucket rotors)	192
For Beckman J2-21 series centrifuges	
JA-10	158
JA-14	137
JA-17	123
JA-18	132
JA-18.1 (25° angle)	112
JA-18.1 (45° angle)	116
JA-20	108
JA-20.1	115
JA-21	102
JCF-Z	89
JCF-Z with small pellet core	81
JE-6B	125
JS-7.5	165
JS-13	142
JS-13.1	140
JV-20	93

continued

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Table A.2C.1 Maximum Rotating Radii for Common Rotors, Grouped by Centrifuge Model
continued

Rotor model[a]	r_{max} (mm)
For Beckman series L7 and L8 ultracentrifuges	
SW 25.1	129.2
SW 28	161.0
SW 28.1	171.3
SW 30	123.0
SW 30.1	123.0
SW 40 Ti	158.8
SW 41 Ti	153.1
SW 50.1	107.3
SW 55 Ti	108.5
SW 60 Ti	120.3
SW 65 Ti	89.0
Type 15	142.1
Type 19	133.4
Type 21	121.5
Type 25	100.4
Type 30	104.8
Type 30.2	94.2
Type 35	104.0
Type 40	80.8
Type 40.3	79.5
Type 42.1	98.6
Type 42.2 Ti	104
Type 45 Ti	103
Type 50	70.1
Type 50 Ti	80.8
Type 50.2 Ti	107.9
Type 50.3 Ti	79.5
Type 50.4 Ti (inner row)	96.4
Type 50.4 Ti (outer row)	111.4
Type 55.2 Ti	100.3
Type 60 Ti	89.9
Type 65	77.7
Type 70 Ti	91.9
Type 70.1 Ti	82.0
Type 75 Ti	79.7
Type 80 Ti	84.0
VAC 50	86.4
VC 53	78.8
VTi 50	86.6
VTi 65	85.4
VTi 65.2	87.9
VTi 80	71.1
For Beckman Airfuge ultracentrifuge	
A-95	17.6
A-100/18	14.6
A-100/30	16.5
A-110	14.7
ACR-90 (2.4-ml liner)	11.8
ACR-90 (3.5-ml liner)	13.4
Batch rotor	14.6
EM-90	13.0
For Beckman TL-100 series ultracentrifuges	
TLA-100	38.9
TLA 100.1	38.9
TLA-100.2	38.9
TLA-100.3	48.3
TLA-45	55.1
TLS-55	76.4
TLV-100	35.7
Miscellaneous centrifuges and rotors[b]	
Clay Adams Dynac	—[c]
Fisher Centrific	113
Fisher Marathon 21K with 4-place rotor	160
IEC Clinical centrifuge with 4-place swinging-bucket rotor	155
IEC general-purpose centrifuge models HN, HN-SII, and Centra-4	—[c]

[a]Sorvall centrifuges and rotors are a product of Du Pont Company Medical Products, Beckman centrifuges are a product of Beckman Instruments, IEC centrifuges are a product of International Equipment Co., Clay Adams Dynac centrifuges are a product of Becton Dickinson Labware, and Fisher centrifuges are a product of Fisher Scientific. For ordering information see *SUPPLIERS APPENDIX*.

[b]These instruments are often loosely referred to as "clinical," "tabletop," or "low-speed" centrifuges.

[c]These instruments accept a wide range of trunnion-ring rotors with variable rotating radii, as well as fixed-angle and swinging-bucket rotors that in turn accept a variety of adapters making it possible to spin different numbers tubes of various sizes. For instance, the commonly used IEC 958 trunnion-ring rotor may be adjusted to radii ranging from 137 to 181 mm, depending on the trunnion-ring chosen. It is therefore necessary to consult the manual for the specific system being used to obtain an accurate speed to RCF conversion.

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Standard Laboratory Equipment

Special equipment is also itemized in the materials list of each protocol. We have not attempted to list all items required for each procedure, but rather have noted those items that might not be readily available in the laboratory or that require special preparation. Listed below are standard pieces of equipment in the modern biochemistry laboratory, i.e., items used extensively in this manual and thus not included in the individual materials lists. See *SUPPLIERS APPENDIX* for contact information for commercial vendors of laboratory equipment.

Autoclave

Balances, analytical and preparative.

Bench protectors, plastic-backed (including "blue pads.")

Centrifuges low-speed (6,000 rpm) and high-speed (20,000 rpm) refrigerated centrifuges and an ultracentrifuge (20,000 to 80,000 rpm) are required for many procedures. At least one microcentrifuge that holds standard 0.5- and 1.5-ml microcentrifuge tubes is essential. It is also useful to have a tabletop swinging-bucket centrifuge with adapters for spinning 96-well microtiter plates.

NOTE: *Centrifuge speeds are provided as × g or as rpm (with example rotor models) throughout the manual. Readers should consult the nomogram in APPENDIX 2C to convert these speeds to their own rotor models.*

Cold room (4°C) or cold box.

Computer (PC or Macintosh) and printer

Containers assortment of plastic and glass dishes for gel and membrane washes.

Darkroom and developing tanks or X-Omat automatic X-ray film developer (Kodak).

Dry ice

Filtration apparatus for collecting acid precipitates on nitrocellulose filters or membrane.

Flasks, glass (e.g., Erlenmeyer, beveled shaker).

Fraction collector

Freezers for –20° and –70°C incubation and storage.

Geiger counter

Gel dryer

Gel electrophoresis equipment at least one full-size horizontal apparatus and one horizontal minigel apparatus, one vertical full-size and minigel apparatus for polyacrylamide protein gels, and specialized equipment for two-dimensional protein gels as required.

Heating blocks thermostat-controlled metal heating blocks that hold test tubes and/or microcentrifuge tubes are very convenient for carrying out enzymatic reactions.

Heat-sealable plastic bags and apparatus

Hoods, chemical and microbiological.

Ice maker

Incubator (37°C) for growing bacteria. We recommend an incubator large enough to hold a "tissue culture" roller drum that can be used to grow 5-ml cultures in standard 18 × 150–mm test tubes. A convenient and durable tube roller is made by New Brunswick Scientific.

Incubator/shaker(s) an enclosed shaker (such as the New Brunswick Controlled Environment Incubator Shaker) that can spin 4-liter flasks is essential for growing 1-liter *E. coli* cultures. A rotary shaking water bath (New Brunswick R76) is useful for growing smaller cultures in flasks.

Light box for viewing gels and autoradiograms.

Liquid nitrogen

Lyophilizers

Magnetic stirrers (with heater is useful) and stir–bars.

Microcentrifuge, Eppendorf-type, maximum speed 12,000 to 14,000 rpm.

Microcentrifuge tubes, 1.5-ml and 0.5-ml.

Microwave oven to melt agar and agarose.

Mortar and pestle

Paper cutter large size, for 46 × 57–cm Whatman paper sheets.

Paper towels

Parafilm

Pasteur pipets and bulbs

pH meter

pH paper

Pipettors that use disposable tips and dispense 1 to 1000 µl. It is best to have a set for

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each full-time researcher, and sets dedicated for radioactive experiments.

Plastic wrap (e.g., Saran Wrap).

Polaroid camera and UV transilluminator for taking photographs of stained gels.

Policemen, rubber or plastic

Power supplies 300-volt power supplies are sufficient for polyacrylamide gels; a 2000- to 3000-volt power supply is needed for some applications.

Racks, to hold test tubes and microcentrifuge tubes.

Radiation shield (Lucite or Plexiglas).

Radioactive waste containers, for liquid and solid waste.

Refrigerator, 4°C.

Safety glasses

Scalpels and blades

Scintillation counter

Scissors

Seal-A-Meal bag sealer or equivalent.

Shakers, orbital and platform, room temperature or 37°C.

Spectrophotometer UV and visible.

Speedvac evaporator (Savant).

Tissue culture equipment CO_2 incubator, phase contrast microscope, liquid nitrogen storage container, and laminar flow hood.

UV light sources, long- and short-wave, stationary or hand-held.

Vortex mixers

Water baths at least two with 80°C capacity.

Water purification equipment such as Milli-Q system (Millipore) or its equivalent.

X-ray film cassettes and intensifying screens

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Commonly Used Reagents

BUFFERS AND STOCK SOLUTIONS

This collection describes the preparation of buffers and reagents used in the manipulation of proteins (see Table A.2E.1). When preparing solutions, use water purified using a Milli-Q apparatus (Millipore) or equivalent and reagents of the highest grade available. Sterilization—by filtration through a 0.22-μm filter—is recommended for most applications.

CAUTION: *Handle strong acids and bases carefully.*

Ammonium acetate, 10 M

Dissolve 385.4 g ammonium acetate in 150 ml H_2O
Add H_2O to 500 ml

Dithiothreitol (DTT), 1 M

Dissolve 15.45 g DTT in 100 ml H_2O
Store at –20°C

EDTA (ethylenediamine tetraacetic acid), 0.5 M (pH 8.0)

Dissolve 186.1 g $Na_2EDTA{\cdot}2H_2O$ in 700 ml H_2O
Adjust pH to 8.0 with 10 M NaOH (~50 ml)
Add H_2O to 1 liter

Begin titrating before the sample is completely dissolved. EDTA, even in the disodium salt form, is difficult to dissolve at this concentration unless the pH is increased to between 7 and 8.

Ethidium bromide, 10 mg/ml

Dissolve 0.2 g ethidium bromide in 20 ml H_2O
Mix well and store at 4°C in dark

CAUTION: *Ethidium bromide is a mutagen and must be handled carefully.*

Table A.2E.1 Molarities and Specific Gravities of Concentrated Acids and Bases

Acid/base	Molecular weight	% by weight	Molarity (approx.)	1 M solution (ml/liter)	Specific gravity
Acids					
Acetic acid (glacial)	60.05	99.6	17.4	57.5	1.05
Formic acid	46.03	90	23.6	42.4	1.205
		98	25.9	38.5	1.22
Hydrochloric acid	36.46	36	11.6	85.9	1.18
Nitric acid	63.01	70	15.7	63.7	1.42
Perchloric acid	100.46	60	9.2	108.8	1.54
		72	12.2	82.1	1.70
Phosphoric acid	98.00	85	14.7	67.8	1.70
Sulfuric acid	98.07	98	18.3	54.5	1.835
Bases					
Ammonium hydroxide	35.0	28	14.8	67.6	0.90
Potassium hydroxide	56.11	45	11.6	82.2	1.447
Potassium hydroxide	56.11	50	13.4	74.6	1.51
Sodium hydroxide	40.0	50	19.1	52.4	1.53

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HCl, 1 M

Mix in the following order:
914.1 ml H_2O
85.9 ml concentrated HCl

KCl, 1 M

74.6 g KCl
H_2O to 1 liter

$MgCl_2$, 1 M

20.3 g $MgCl_2 \cdot 6H_2O$
H_2O to 100 ml

$MgSO_4$, 1 M

24.6 g $MgSO_4 \cdot 7H_2O$
H_2O to 100 ml

NaCl, 5 M

292 g NaCl
H_2O to 1 liter

NaOH, 10 M

Mix in the following order:
476 ml H_2O
524 ml 50% (v/v) NaOH
Alternatively:
Dissolve 400 g NaOH in 450 ml H_2O
Add H_2O to 1 liter

Phenol, buffered

8-hydroxyquinoline
Liquefied phenol, redistilled
50 mM Tris base (unadjusted pH ~10.5)
50 mM Tris·Cl, pH 8.0 (see recipe)
TE buffer, pH 8.0 (see recipe)

Add 0.5 g of 8-hydroxyquinoline to a 2-liter glass beaker containing a stir bar. Gently pour in 500 ml of liquefied phenol or melted crystals of redistilled phenol (melted in a water bath at 65°C). The phenol will turn yellow due to the 8-hydroxyquinoline, which is added as an antioxidant. Add 500 ml of 50 mM Tris base. Cover the beaker with aluminum foil and stir 10 min at low speed with magnetic stirrer at room temperature. Let phases separate at room temperature. Gently decant the top (aqueous) phase into a suitable waste receptacle. Remove what cannot be decanted with a 25-ml glass pipet and a suction bulb. Add 500 ml of 50 mM Tris·Cl, pH 8.0. Repeat equilibration with 500 ml of 50 mM Tris·Cl, pH 8.0, twice. The pH of the phenol phase can be checked with indicator paper and should be 8.0. If it is not, repeat equilibration until this pH is obtained. Add 250 ml of 50 mM Tris·Cl, pH 8.0, or TE buffer, pH 8.0, and store at 4°C in brown glass bottles or clear glass bottles wrapped in aluminum foil.

Phenol prepared with 8-hydroxyquinoline as an antioxidant can be stored ≤2 months at 4°C.

Phenol must be redistilled before use, because oxidation products of phenol can damage and introduce breaks into nucleic acid chains. Redistilled phenol is commercially available. Regardless of the source, the phenol must be buffered before use.

CAUTION: *Phenol can cause severe burns to skin and damage clothing. Gloves, safety glasses, and a lab coat should be worn whenever working with phenol, and all manipulations should be carried out in a fume hood. A glass receptacle should be available exclusively for disposing of used phenol and chloroform.*

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Table A.2E.2 Preparation of 0.1 M Sodium and Potassium Acetate Buffers[a]

Desired pH	Solution A (ml)	Solution B (ml)
3.6	46.3	3.7
3.8	44.0	6.0
4.0	41.0	9.0
4.2	36.8	13.2
4.4	30.5	19.5
4.6	25.5	24.5
4.8	20.0	30.0
5.0	14.8	35.2
5.2	10.5	39.5
5.4	8.8	41.2
5.6	4.8	45.2

[a]Adapted by permission from CRC, 1975.

Table A.2E.3 Preparation of 0.1 M Sodium and Potassium Phosphate Buffers[a]

Desired pH	Solution A (ml)	Solution B (ml)	Desired pH	Solution A (ml)	Solution B (ml)
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	90.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

[a]Adapted by permission from CRC, 1975.

Phosphate-buffered saline (PBS)

10× stock solution, 1 liter:
80 g NaCl
2 g KCl
11.5 g $Na_2HPO_4 \cdot 7H_2O$
2 g KH_2PO_4

Working solution, pH ~7.3:
137 mM NaCl
2.7 mM KCl
4.3 mM $Na_2HPO_4 \cdot 7H_2O$
1.4 mM KH_2PO_4

Potassium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid/liter (0.2 M)
Solution B: 19.6 g potassium acetate ($KC_2H_3O_2$)/liter (0.2 M)

Referring to Table A.2E.2 for desired pH, mix the indicated volumes of solutions A and B, then dilute with H_2O to 100 ml.

This may be made as a 5- or 10-fold concentrate by scaling up the amount of potassium acetate in the same volume. Acetate buffers show concentration-dependent pH changes, so check concentrate pH by diluting an aliquot to the final concentration.

To prepare buffers with pH intermediate between the points listed in Table A.2E.2, prepare closest higher pH, then titrate with solution A.

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Potassium phosphate buffer, 0.1 M

Solution A: 27.2 g KH_2PO_4 per liter (0.2 M)
Solution B: 45.6 g $K_2HPO_4 \cdot 3H_2O$ per liter (0.2 M)

Referring to Table A.2E.3 for desired pH, mix the indicated volumes of solutions A and B, then dilute with H_2O to 200 ml.

This may be made as a 5- or 10-fold concentrate by scaling up the amount of potassium phosphate in the same volume. Phosphate buffers show concentration-dependent pH changes, so check concentrate pH by diluting an aliquot to the final concentration.

SDS, 20%

Dissolve 20 g SDS (sodium dodecyl sulfate or sodium lauryl sulfate) in H_2O to 100 ml total with stirring (it may be necessary to heat the solution slightly to fully dissolve the powder). Filter sterilize using a 0.45-μm filter.

Sodium acetate, 3 M

Dissolve 408 g sodium acetate·$3H_2O$ in 800 ml H_2O
Add H_2O to 1 liter
Adjust pH to 4.8 or 5.2 (as desired) with 3 M acetic acid

Sodium acetate buffer, 0.1 M

Solution A: 11.55 ml glacial acetic acid/liter (0.2 M)
Solution B: 27.2 g sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$)/liter (0.2 M)

Referring to Table A.2E.2 for desired pH, mix the indicated volumes of solutions A and B, then dilute with H_2O to 100 ml. (See Potassium acetate buffer recipe for further details.)

Sodium phosphate buffer, 0.1 M

Solution A: 27.6 g $NaH_2PO_4 \cdot H_2O$ per liter (0.2 M)
Solution B: 53.65 g $Na_2HPO_4 \cdot 7H_2O$ per liter (0.2 M)

Referring to Table A.2E.3 for desired pH, mix the indicated volumes of solutions A and B, then dilute with H_2O to 200 ml. (See Potassium phosphate buffer recipe for further details.)

SSC (sodium chloride/sodium citrate), 20×

3 M NaCl (175 g/liter)
0.3 M Na_3citrate·$2H_2O$ (88 g/liter)
Adjust pH to 7.0 with 1 M HCl

TAE (Tris/acetate/EDTA) electrophoresis buffer

50× stock solution:
242 g Tris base
57.1 ml glacial acetic acid
37.2 g $Na_2EDTA \cdot 2H_2O$
H_2O to 1 liter

Working solution, pH ~8.5:
40 mM Tris·acetate
2 mM $Na_2EDTA \cdot 2H_2O$

TBE (Tris/borate/EDTA) electrophoresis buffer

10× stock solution, 1 liter:
108 g Tris base (890 mM)
55 g boric acid (890 mM)
40 ml 0.5 M EDTA, pH 8.0 (see recipe; 20 mM)

TE buffer

10 mM Tris·Cl, pH 7.4, 7.5, or 8.0 (or other pH; see recipe)
1 mM EDTA, pH 8.0

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Tris·Cl [tris(hydroxymethyl)aminomethane], 1 M

Dissolve 121 g Tris base in 800 ml H_2O
Adjust to desired pH with concentrated HCl
Mix and add H_2O to 1 liter

NOTE: *The pH of Tris buffers changes significantly with temperature, decreasing approximately 0.028 pH units per 1°C. Tris-buffered solutions should be adjusted to the desired pH at the temperature at which they will be used. Because the pK_a of Tris is 8.08, Tris should not be used as a buffer below pH ~7.2 or above pH ~9.0.*

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Use of Protein Folding Reagents

DENATURANTS

Chemical agents that denature proteins are used to extract proteins from inclusion bodies as well as to study the conformation of purified proteins (Pace, 1986). The most common denaturants are guanidine hydrochloride (guanidine·HCl) and urea. These reagents and methods for their purification are described in this section.

Guanidine Hydrochloride

High-purity guanidine·HCl (also referred to as guanidinium chloride) can be purchased from several vendors as a crystalline solid or concentrated solution (usually 8 M) in water. Because dissolution of the salt is an endothermic process, when preparing concentrated solutions from the solid salt it is convenient to heat the mixture (in a glass container) in a domestic microwave oven. When the best grades of commercial guanidine·HCl (>99% pure) are used, a 6 M solution will be clear and colorless. Concentrated solutions made from practical-grade guanidine·HCl (usually 80% to 90% cheaper) may appear slightly hazy and be colored light brown. For large-scale preparative work, where the purchase of sufficient high-purity reagent would be prohibitive, solutions of practical-grade guanidine·HCl can be cleaned up adequately by filtration with 0.45- to 0.5-μm filters followed by decolorization with activated charcoal. Other applications, such as folding experiments (especially those involving spectroscopic monitoring), require higher-quality reagent; in such cases it is necessary to recrystallize the salt or purchase the ultrapure grade. Methods for purifying guanidine·HCl by recrystallization have been described by Nozaki (1972).

Because guanidine·HCl is hydroscopic, for critical studies, such as conformational analysis, accurate measurements of molarity should be made using a refractometer (e.g., the Abbe-3L refractometer from Milton Roy; see *SUPPLIERS APPENDIX*). Using Table A.3A.1 as a guideline for quantities, guanidine·HCl and water (or buffer) can be weighed in a container to give the approximate molarities indicated. A table of the refractive indexes of guanidine·HCl solutions from 0.057 M to 8.51 M (in increments of ~0.06 M) at 25°C is provided by Nozaki (1972).

There have been no reports, at least to the author's knowledge, of specific chemical modification of proteins resulting from exposure to guanidine·HCl or the contaminants found in the commercially purchased forms. However, the common contaminant ammeline, which is characterized by strong UV absorbance, may inhibit certain enzymes (Nozaki and Tanford, 1967; Nozaki, 1972, and references therein).

Guanidine·HCl solutions are stable at room temperature for several days (for longer periods, store at 4°C). Solutions are stable over normal working pH ranges—i.e., pH 2.0 to 10.5. At alkaline pH (>11), biguanidine may form (Nozaki and Tanford, 1967).

Urea

High-purity (>99%) urea can be purchased from many different vendors and is only approximately twice as expensive as practical-grade material. Urea solutions slowly decompose to form ammonium cyanate, which reacts readily with various protein functional groups. An 8 M solution of urea incubated at room temperature >pH 7.0 will contain ~4 mM cyanate after 7 days and ~20 mM at equilibrium.

Table A.3A.1 Properties of Urea and Guanidine·HCl Solutions

Property	Urea	Guanidine·HCl
Molecular weight	60.06	95.53
Solubility (at 25°C)[a]	10.49 M	8.54 M
Melting point (°C)	133-137	186.5-188.5
A_{260} (6 M in water)	<0.06	<0.03

[a]Solubilities at 5°C: urea, ~8 M; guanidine·HCl, ≥8 M.

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Table A.3A.2 Preparation of Urea and Guanidine·HCl Solutions

Molarity	Urea			Guanidine·HCl		
	Quantity[a]	Vol (ml)[b]	Concentration (g/liter)[c]	Quantity[a]	Vol (ml)[b]	Concentration (g/liter)[c]
1 M	0.63	10.52	60.06	1.03	10.82	95.53
2 M	1.32	11.04	120.12	2.23	11.71	191.06
3 M	2.09	11.61	180.18	3.65	12.76	286.59
4 M	2.93	12.24	240.24	5.36	14.05	382.12
5 M	3.88	12.96	300.30	7.45	15.63	477.65
6 M	4.95	13.76	360.36	10.09	17.63	573.18
7 M	6.16	14.68	420.42	13.51	20.23	668.71
8 M	7.54	15.73	480.48	18.14	23.77	764.24
9 M	9.15	16.95	540.54	—	—	—
10 M	11.02	18.38	600.60	—	—	—

[a]Given weight of reagent added to 10 g water to generate a solution of the indicated molarity.

[b]The final volume resulting from the addition of indicated amount of reagent to 10 g water was determined using the specific gravity of the solution calculated as described by Kawahara and Tanford (1966). Volume estimates can also be made by assuming 1 g of urea or guanidine·HCl increases the volume by 0.763 ml (i.e., the partial specific volume = 0.763). The density of water at 25°C is 0.9971 (10 g = 10.029 ml).

[c]Weight of reagent made up with water or buffer to a final volume of 1 liter.

Formation of cyanate occurs faster at higher temperatures (e.g., equilibrium is reached in 30 min at 100°C), and it may therefore be helpful to keep the solution cold. Cyanate reacts readily with the amino, sulfhydryl, imidazole, tyrosyl, and carboxyl groups of proteins. Only the reaction with amino groups results in stable products at alkaline pH (Means and Feeney, 1971). The carbamylation of α-amino (N-terminal) and ε-amino groups (lysine side chains) results in the formation of a neutral product, homocitrulline; hence there is a loss of one positive charge for each residue modified.

The standard method for removing cyanate is deionization using a mixed-bed ion-exchange resin (e.g., AG 501-X8, Bio-Rad; see *SUPPLIERS APPENDIX*). The batch method (adding resin to the solution, incubating, then filtering) is recommended for volumes <1 liter, whereas the column method (using resin packed in a chromatography column) is best for larger volumes (see Bio-Rad Bulletin 1825 and *UNIT 8.2*). Cyanate removal can be monitored by determining the solution conductivity; there are also simple specific assays available (Means and Feeney, 1972). For a detailed purification method, involving both recrystallization and deionization, see Prakash et al. (1981). Cyanate can also be removed by acidification to pH 2 with HCl, followed by a 1-hr incubation during which the cyanate breaks down to carbon dioxide and ammonium ions, and neutralization with Tris base. The solution can then be used as is.

For most applications it is recommended that solutions be made with ultrapure urea and used within 24 hr. A scavenger should be included to react with slowly forming cyanate; buffers containing primary amines, such as Tris and glycine, are suitable for this purpose. It should be noted that urea may increase the measured pH of aqueous solutions (see Bull et al., 1964).

As with guanidine·HCl, refractometry can be used to accurately determine the concentration of solutions, although solutions prepared by weight (Table A.3A.2) are normally accurate enough for most applications (Pace, 1986).

Effectiveness

According to Pace (1986) and references therein, guanidine·HCl is generally 1.5 to 2.5 times more effective per mole as a protein denaturant than urea.

Other Reagents

Guanidine thiocyanate is another denaturant of interest that is often used to elute proteins from immunoaffinity columns. Although guanidine thiocyanate is rarely used in preparative folding of recombinant proteins or in conformational analysis of proteins in general, it is

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Table A.3A.3 Reduced and Oxidized Sulfhydryl Reagents

Reagent	MW[a]	MP (°C)	Absorbance (nm) (liter/mol/cm)[e]	SH[f] pK_a	E'_o (mV)[g]
GSH	307.33	192-195	260 (5.8) 280 (2.3)	8.63 (α) 9.45 (β)	–240
GSSG	612.63	178	250 (342) 260 (271) 280 (111) 300 (30)	—	
2-ME[b]	78.13	—	>260 (≈ 0, if pure)	9.5	
HED[c]	154.25	25-27	245 λ_{max} (380) 260 (310) 280 (200)	—	
DTT	154.25	42-44	>270 (≈ 0)	8.3 9.5	–330
Oxidized DTT[d]	152.20	132	283 λ_{max} (273) 310 (110)	—	

[a]Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione (γ-L-glutamyl-L-cysteinylglycine, anionic thiol); GSSG, oxidized glutathione; HED, 2-hydroxyethyl disulfide; MP, melting point; MW, molecular weight.

[b]Pure liquid is 14.3 M (density = 1.114 g/ml) with a boiling point of 157° – 158°C.

[c]Liquid is 8.2 M (density = 1.261 g /ml).

[d]Oxidized DTT (dihydroxy-1,2-dithiane) can be prepared from DTT by oxidation with ferricyanide (Cleland, 1964). Commercial material can be purified according to Creighton (1984).

[e]The values in parenthesis are the molar absorbencies at the wavelengths indicated.

[f]In amino thiols, the pK_a of the amine and thiol groups overlap; relevant pK_a's are indicated as α and β.

[g]The standard redox potential (at pH 7, 25°C); refers to the reduction potential for thiol-disulfide pairs: $2RSH = R\text{-}S\text{-}S\text{-}R + 2H^{++} + 2e^-$. Note that the lower the E'_o, the stronger the reductant—e.g., DTT is a better reductant than GSH.

2.5 to 3.5 times more effective than guanidine·HCl, and is available (albeit expensive) in high purity.

Other chaotropic reagents, mainly inorganic salts, are briefly reviewed and referenced in the Commentary section of *UNIT 6.3*. As there are few (if any) reports in the literature regarding the use of these reagents in solubilizing inclusion bodies, detailed discussion of their properties is not included. Future updates of this section will, however, address any important new reagents.

SULFHYDRYL REAGENTS AND OXIDO-SHUFFLING SYSTEMS

Sulfhydryl reagents (reducing agents) are used to prevent the oxidation of protein thiol groups. When proteins are extracted from inclusion bodies it is standard practice to include a reducing agent to prevent random oxidation of cysteine residues. Denatured proteins are subsequently folded under conditions that favor native disulfide formation. This process, referred to as oxidative folding, is carried out using "oxido-shuffling" (or oxidative regeneration) systems consisting of low-molecular-weight thiol/disulfide pairs (*UNITS 6.4 & 6.5*). The thiols and disulfides commonly used to reduce and oxidize recombinant proteins are shown in Table A.3A.3 and discussed in this section. Further details on these and other reducing agents are reviewed by Jocelyn (1987).

Dithiothreitol

Dithiothreitol (DTT), also known as Cleland's reagent, is the preferred reductant for protein reduction. DTT and its isomer dithioerythritol (DTE)—which can also be used as a protein reductant—have similar properties (and prices), except that at lower pH the former is more effective. This is because the pK_a values for DTE are higher (9.0 and 9.9) than those of DTT (8.3 and 9.5); hence, a higher pH is required to ionize DTE to its functional form.

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The reducing capacity of both reagents increases greatly over the pH range 7 to 9.5. On the other hand, the reagents are quenched by acidification to <pH 3.0.

DTT is very soluble in water, and stock solutions of 0.1 to 0.2 M can be prepared and stored at 4°C for a day or so or frozen for longer periods. For critical work, solid DTT should be added to solvents and buffers immediately before use to achieve a final working concentration in the range of 1 to 20 mM. Because DTT complexes metal ions, EDTA is usually included in the reduction buffer. Air oxidation of DTT solution can be monitored by measuring the increase in absorbance at 283 nm (see Table A.3A.3).

2-Mercaptoethanol

2-Mercaptoethanol (2-ME) is also commonly used as a protein reducing agent. 2-ME is a volatile liquid that should be stored at 4°C and dispensed under a fume hood. A 100-mM stock solution of 2-ME can be made by diluting 6.99 ml of the reagent with solvent to 1 liter. 2-ME is not as potent a reductant as DTT, as it has a higher pK_a (Table A.3A.3), and is used in the concentration range of 5 to 100 mM. Air oxidation of 2-ME is best monitored by measuring the increase in absorbance at 260 nm (Table A.3A.3). Freshly opened bottles contain 0.1% to 6.7% of the oxidation product 2-hydroxyethyl disulfide (HED; Wetlaufer et al., 1987).

Glutathione

Glutathione (GSH) and its oxidized form GSSG are present in most cells at concentrations of 1 to 10 mM. In *E. coli*, the ratio of GSH/GSSG is 50:1 to 200:1 (Hwang et al., 1992), and the system is responsible for maintaining cytoplasmic proteins in a reduced state. GSH is not normally used as a reductant in analytical protein chemistry—its main utility is as a component of oxidative redox buffers (see discussion of Oxido-Shuffling Systems). However, GSH can be used to maintain unfolded protein in inclusion-body extracts in the reduced state (analogous to its role in vivo).

Stock solutions of 0.1 M GSH can be prepared in water or the reagent can be directly added to buffers. In either case the pH should be adjusted with base to the required pH.

Oxido-Shuffling Systems

The commonly used oxido-shuffling systems used in folding recombinant proteins are GSH/GSSG, 2-ME/HED, and DTT/oxidized DTT (for reviews, see Creighton, 1984, and Wetlaufer, 1984), which are shown in Table A.3A.3. All six reagents are commercially available (the scarcest, HED, can be obtained from Aldrich; see *SUPPLIERS APPENDIX*) and are normally used as purchased, without further purification.

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Dialysis

Conventional dialysis separates small molecules from large molecules by allowing diffusion of only the small molecules through selectively permeable membranes. Dialysis is usually used to change the salt (small-molecule) composition of a macromolecule-containing solution. The solution to be dialyzed is placed in a sealed dialysis membrane and immersed in a selected buffer; small solute molecules then equilibrate between the sample and the dialysate. Concomitant with the movement of small solutes across the membrane, however, is the movement of solvent in the opposite direction. This can result in some sample dilution (usually <50%).

This section describes dialysis of large- and small-volume samples using cellulose membranes with pore sizes designed to exclude molecules above a selected molecular weight (Basic and Alternate Protocols). A Support Protocol describes preparation of membranes for dialysis.

LARGE-VOLUME DIALYSIS

BASIC PROTOCOL

This protocol describes the use of membranes, prepared using the support protocol, for dialysis of samples in large, easily handled volumes, typically 0.1 to 500 ml.

Materials

Dialysis membrane (see Support Protocol)
Clamps (Spectrapor Closures, Spectrum, or equivalent)
Macromolecule-containing sample to be dialyzed
Appropriate dialysis buffer

1. Remove dialysis membrane from ethanol storage solution and rinse with distilled water. Secure clamp to one end of the membrane or knot one end with double-knots.

   Always use gloves to handle the dialysis membrane because the membrane is susceptible to cellulolytic microorganisms.

   See support protocol for a discussion of commercially available membranes and preparation and storage procedures.

   Clamps are less likely to leak than knots, but either type of closure should be carefully tested as described in steps 2 and 3.

2. Fill membrane with water or buffer, hold the unclamped end closed, and squeeze membrane. A fine spray of liquid indicates a pinhole in the membrane; discard and try a new membrane.

   Pinholes are rare but cause traumatic sample loss. Hard squeezing will cause some liquid to seep from the bag; this is normal.

3. Replace the water or buffer in dialysis membrane with the macromolecule-containing sample and clamp the open end. Again, squeeze to check the integrity of the membrane and clamps.

   If dialyzing a concentrated or high-salt sample, leave some space in the clamped membrane; there will be a net flow of water into the sample, and if sufficient pressure builds up the membrane can burst.

4. Immerse dialysis membrane in a beaker or flask containing a large volume (relative to the sample) of the desired buffer. Dialyze at least 3 hr at the desired temperature with gentle stirring of the buffer.

   Dialysis rates are dependent on membrane pore size, sample viscosity, and ratio of membrane surface to sample volume. Temperature has little effect on dialysis rate, but

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low temperatures (i.e., 4°C) are usually chosen to enhance macromolecule stability. Common salts will equilibrate across a 15,000-MWCO membrane (see Support Protocol) in ~3 hr with stirring.

Some compounds, especially detergents above their critical micelle concentration (CMC), dialyze very slowly.

5. Change dialysis buffer as necessary.

   Usually two to three dialysis buffer changes are sufficient. For example, when 100 mM Tris·Cl is removed from a protein for sequence analysis or other amino-reactive chemistry, two equilibriums against a 1000-fold volume excess of buffer will decrease the Tris concentration 10^6-fold, to 100 nM; three dialyses will decrease the concentration to 100 pM.

6. Remove dialysis membrane from the buffer. Hold the membrane vertically and remove excess buffer trapped in end of membrane outside upper clamp. Release upper clamp and remove the sample with a Pasteur pipet.

ALTERNATE PROTOCOL

SMALL-VOLUME DIALYSIS

For solution volumes less than ~100 µl, the use of dialysis membrane as described above can result in unacceptable sample loss. The method described below can easily dialyze volumes as small as 10 µl. The sample is held in a 0.5-ml microcentrifuge tube, with dialysis membrane covering the open end of the tube.

Additional Materials

0.5-ml microcentrifuge tube
Cork borer

1. Cut a hole in the cap of a 0.5-ml microcentrifuge tube using a cork borer. Be sure that there are no rough edges on the inside of the cap (which will be in contact with the dialysis membrane).

   The success of this protocol is dependent on a tight-fitting cap, so make sure the cap is well-seated. On the other hand, an excessively tight-fitting cap can rip the membrane when it is inserted. Sarstedt tubes work well.

   Alternatively, some researchers use a microcentrifuge tube without a cap. The sample is placed in the microcentrifuge tube, a dialysis membrane is placed over the top of the tube, and the membrane is secured with a rubber band. With this method, however, there is a risk that a small sample—e.g., 10 to 20 µl—may be lost around the edge of the tube.

2. Place sample in the microcentrifuge tube, cover tube opening with dialysis membrane, and snap on the tube cap in which a hole has been cut.

   This places a fair amount of stress on the dialysis membrane, so use a fairly thick membrane and make sure there are no rough edges on the cap.

3. To get the sample in contact with the dialysis membrane, *gently* centrifuge the microcentrifuge tube in an inverted position in a tabletop centrifuge.

4. Keep the tube inverted, immerse it in dialysis buffer, and anchor it so that it will remain inverted and immersed.

   This is easily done by inserting tubes in a styrofoam or foam sheet (e.g., Fisherbrand foam microcentrifuge tube rack) and placing the inverted tubes on the surface of the dialysis buffer.

5. Use a bent Pasteur pipet to blow out any air bubbles trapped under the cap to allow dialysis buffer to contact the membrane.

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This step is usually not necessary if the membrane/rubber band method is used (see step 1 annotation).

6. Stir the dialysis buffer and dialyze at an appropriate temperature for at least 3 hr (see Basic Protocol, step 4).

7. To recover the sample, remove microcentrifuge tube from the buffer and centrifuge briefly right-side-up.

Commercially available alternatives to this method include use of individual hollow-fiber filters from Spectrum (with sample capacities of 1 to 140 µl), and many different microdialysis machines, both single-sample and multisample (Spectrum, Cole-Parmer, Hoefer Pharmacia, and others).

SELECTION AND PREPARATION OF DIALYSIS MEMBRANE

SUPPORT PROTOCOL

Dialysis membranes are available in a number of thicknesses and pore sizes. Thicker membranes are tougher, but restrict solute flow and reach equilibrium more slowly. Pore size is defined by "molecular weight cutoff" (MWCO)—i.e., the size of the smallest particle that cannot penetrate the membrane. The MWCO designation should be used only as a very rough guide; if accurate MWCO information is required, it should be determined empirically (see Craig, 1967, for a discussion of parameters affecting MWCO). Knowledge of the precise MWCO is usually not required, however; it is necessary only to use a membrane with a pore size that is much smaller than the macromolecule of interest. For most plasmid and protein dialyses, a MWCO of 12,000 to 14,000 is appropriate, whereas a MWCO of 3500, 2000, or even 1000 is appropriate for peptides.

Most dialysis membranes are made of derivatives of cellulose. They come in a wide variety of MWCO values, ranging from 500 to 500,000, and also vary in cleanliness, sterility, and cost. Spectrum has the most impressive inventory. The least expensive membranes come dry on rolls; these contain glycerol to keep them flexible as well as residual sulfides and traces of heavy metals from their manufacture. If glycerol, sulfur compounds, or small amounts of heavy metals are problematic, cleaner membranes should be purchased or membranes should be prepared as described below. Protein dialysis should only be done with clean membranes.

Additional Materials

10 mM sodium bicarbonate
10 mM Na_2EDTA, pH 8.0
20% to 50% (v/v) ethanol

1. Remove membrane from the roll and cut into usable lengths (usually 8 to 12 in.).

Always use gloves to handle dialysis membrane, as it is susceptible to a number of cellulolytic microorganisms. Membrane is available as sheets or preformed tubing.

2. Wet membrane and boil it for several minutes in a large excess of 10 mM sodium bicarbonate.

3. Boil several minutes in 10 mM Na_2EDTA. Repeat.

Boiling speeds up the treatment process but is not necessary. A 30-min soak with some agitation can substitute for the boiling step.

4. Wash several times in distilled water.

5. Store at 4°C in 20% to 50% ethanol to prevent growth of cellulolytic microorganisms.

Alternatively, bacteriostatic agents (e.g., sodium azide or sodium cacodylate) may be used for storage; however, ethanol is preferred for ease and convenience.

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Describe the theory and practice of dialysis and diffusion.

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Techniques for Mammalian Cell Tissue Culture

Tissue culture technology has found wide application in biological research. Monolayer cell cultures are utilized in cytogenetic, biochemical, and molecular laboratories for research as well as diagnostic studies. In most cases, cells or tissues must be grown in culture for days or weeks to obtain sufficient numbers of cells for analysis. Maintenance of cells in long-term culture requires strict adherence to aseptic technique to avoid contamination and potential loss of valuable cell lines. The first section of this appendix discusses basic principles of sterile technique.

An important factor influencing the growth of cells in culture is the choice of tissue culture medium. Many different recipes for tissue culture media are available and each laboratory must determine which medium best suits their needs. Individual laboratories may elect to use commercially prepared medium or prepare their own. Commercially available medium can be obtained as a sterile and ready-to-use liquid, in a concentrated liquid form, or in a powdered form. Besides providing nutrients for growing cells, medium is generally supplemented with antibiotics, fungicides, or both to inhibit contamination. The second section of this appendix discusses medium preparation.

As cells grown in monolayer reach confluency, they must be subcultured or passaged. Failure to subculture confluent cells results in reduced mitotic index and eventually in cell death. The first step in subculturing is to detach cells from the surface of the primary culture vessel by trypsinization or mechanical means. The resultant cell suspension is then subdivided, or reseeded, into fresh cultures. Secondary cultures are checked for growth, fed periodically, and may be subsequently subcultured to produce tertiary cultures, etc. The time between passaging cells varies with the cell line and depends on the growth rate.

The Basic Protocol describes subculturing of a monolayer culture grown in petri dishes or flasks. Support Protocols 1 to 4 describe freezing of monolayer cells, thawing and recovery of cells, counting cells using a hemacytometer, and preparing cells for transport. The Alternate Protocol describes freezing suspension cells.

STERILE TECHNIQUE

It is essential that sterile technique be maintained when working with cell cultures. Aseptic technique involves a number of precautions to protect both the cultured cells and the laboratory worker from infection. The laboratory worker must realize that cells handled in the lab are potentially infectious and should be handled with caution. Protective apparel such as gloves, lab coats or aprons, and eyewear should be worn when appropriate (Knutsen, 1991). Care should be taken when handling sharp objects such as needles, scissors, scalpel blades, and glass that could puncture the skin. Sterile disposable plastic supplies may be used to avoid the risk of broken or splintered glass (Rooney and Czepulkowski, 1992).

Frequently, specimens received in the laboratory are not sterile, and cultures prepared from these specimens may become contaminated with bacteria, fungus, or yeast. The presence of microorganisms can inhibit growth, kill cell cultures, or lead to inconsistencies in test results. The contaminants deplete nutrients in the medium and may produce substances that are toxic to cells. Antibiotics (penicillin, streptomycin, kanamycin, or gentamycin) and fungicides (amphotericin B or mycostatin) may be added to tissue

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culture medium to combat potential contaminants (see Table A.3C.1). An antibiotic/antimycotic solution or lyophilized powder that contains penicillin, streptomycin, and amphotericin B is available from Sigma. The solution can be used to wash specimens prior to culture and can be added to medium used for tissue culture. Similar preparations are available from other suppliers.

All materials that come into direct contact with cultures must be sterile. Sterile disposable dishes, flasks, pipets, etc., can be obtained directly from manufacturers. Reusable glassware must be washed, rinsed thoroughly, then sterilized by autoclaving or by dry heat before reusing. With dry heat, glassware should be heated 90 min to 2 hr at 160°C to ensure sterility. Materials that may be damaged by very high temperatures can be autoclaved 20 min at 120°C and 15 psi. All media, reagents, and other solutions that come into contact with the cultures must also be sterile; medium may be obtained as a sterile liquid from the manufacturer, autoclaved if not heat-sensitive, or filter sterilized. Supplements can be added to media prior to filtration, or they can be added aseptically after filtration. Filters with 0.20- to 0.22-μm pore size should be used to remove small gram-negative bacteria from culture media and solutions.

Contamination can occur at any step in handling cultured cells. Care should be taken when pipetting media or other solutions for tissue culture. The necks of bottles and flasks, as well as the tips of the pipets, should be flamed before the pipet is introduced into the bottle. If the pipet tip comes into contact with the benchtop or any other nonsterile surface, it should be discarded and a fresh pipet obtained. Forceps and scissors used in tissue culture can be rapidly sterilized by dipping in 70% alcohol and flaming.

Although tissue culture work can be done on an open bench if aseptic methods are strictly enforced, many labs prefer to perform tissue culture work in a room or low-traffic area reserved specifically for that purpose. At the very least, biological safety cabinets are recommended to protect the cultures as well as the laboratory worker. In a laminar flow hood, the flow of air protects the work area from dust and contamination and acts as a barrier between the work surface and the worker. Many different styles of safety hoods are available and the laboratory should consider the types of samples being processed and the types of potential pathogenic exposure in making their selection. Manufacturer recommendations should be followed regarding routine maintenance checks on air flow and filters. For day-to-day use, the cabinet should be turned on for at least 5 min prior to beginning work. All work surfaces both inside and outside of the hood should be kept clean and disinfected daily and after each use.

Some safety cabinets are equipped with ultraviolet (UV) lights for decontamination of work surfaces. However, use of UV lamps is no longer recommended, as it is generally ineffective (Knutsen, 1991). UV lamps may produce a false sense of security as they maintain a visible blue glow long after their germicidal effectiveness is lost. Effectiveness diminishes over time as the glass tube gradually loses its ability to transmit short UV wavelengths, and it may also be reduced by dust on the glass tube, distance from the work surface, temperature, and air movement. Even when the UV output is adequate, the rays must directly strike a microorganism in order to kill it; bacteria or mold spores hidden below the surface of a material or outside the direct path of the rays will not be destroyed. Another rule of thumb is that anything that can be seen cannot be killed by UV. UV lamps will only destroy microorganisms such as bacteria, virus, and mold spores; they will not destroy insects or other large organisms (Westinghouse Electric Company, 1976). The current recommendation is that work surfaces be wiped down with ethanol instead of relying on UV lamps, although some labs use the lamps in addition to ethanol wipes to decontaminate work areas. A special metering device is available to measure the output

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of UV lamps, and the lamps should be replaced when they fall below the minimum requirements for protection (Westinghouse Electric Company, 1976).

Cultures should be checked routinely for contamination. Indicators in the tissue culture medium change color when contamination is present: for example, medium that contains phenol red changes to yellow because of increased acidity. Cloudiness and turbidity are also observed in contaminated cultures. Once contamination is confirmed with a microscope, infected cultures are generally discarded. Keeping contaminated cultures increases the risk of contaminating other cultures. Sometimes a contaminated cell line can be salvaged by treating it with various combinations of antibiotics and antimycotics in an attempt to eradicate the infection (e.g., see Fitch et al., 1991). However, such treatment may adversely affect cell growth and is often unsuccessful in ridding cultures of contamination.

PREPARING CULTURE MEDIUM

Choice of tissue culture medium comes from experience. An individual laboratory must select the medium that best suits the type of cells being cultured. Chemically defined media are available in liquid or powdered form from a number of suppliers. Sterile, ready-to-use medium has the advantage of being convenient, although it is more costly than other forms. Powdered medium must be reconstituted with tissue culture grade water according to manufacturer's directions. Distilled or deionized water is not of sufficiently high quality for medium preparation; double- or triple-distilled water or commercially available tissue culture water should be used. The medium should be filter-sterilized and transferred to sterile bottles. Prepared medium can generally be stored ≤1 month in a 4°C refrigerator. Laboratories using large volumes of medium may choose to prepare their own medium from standard recipes. This is the most economical approach, but it is time-consuming.

Basic media such as Eagle's minimal essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), Glasgow modified Eagle medium (GMEM), and RPMI 1640 and Ham F10 nutrient mixtures (e.g., Life Technologies) are composed of amino acids, glucose, salts, vitamins, and other nutrients. A basic medium is supplemented by addition of L-glutamine, antibiotics (typically penicillin and streptomycin sulfate), and usually serum to formulate a "complete medium." Where serum is added, the amount is indicated as a percentage of fetal bovine serum (FBS) or other serum. Some media are also supplemented with antimycotics, nonessential amino acids, various growth factors, and/or drugs that provide selective growth conditions. Supplements should be added to medium prior to sterilization or filtration, or added aseptically just before use.

The optimum pH for most mammalian cell cultures is 7.2 to 7.4. Adjust pH of the medium as necessary after all supplements are added. Buffers such as bicarbonate and HEPES are routinely used in tissue culture medium to prevent fluctuations in pH that might adversely affect cell growth. HEPES is especially useful in solutions used for procedures that do not take place in a controlled CO_2 environment.

Most cultured cells will tolerate a wide range of osmotic pressure and an osmolarity between 260 and 320 mOsm/kg is acceptable for most cells. The osmolarity of human plasma is ~290 mOsm/kg, and this is probably the optimum for human cells in culture as well (Freshney, 1993).

Fetal bovine serum (FBS; sometimes known as fetal calf serum, FCS) is the most frequently used serum supplement. Calf serum, horse serum, and human serum are also used; some cell lines are maintained in serum-free medium (Freshney, 1993). Complete

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Table A.3C.1 Working Concentrations of Antibiotics and Fungicides for Mammalian Cell Culture

Additive	Final concentration
Penicillin	50–100 U/ml
Streptomycin sulfate	50–100 µg/ml
Kanamycin	100 µg/ml
Gentamycin	50 µg/ml
Mycostatin	20 µg/ml
Amphotericin B	0.25 µg/ml

medium is supplemented with 5% to 30% (v/v) serum, depending on the requirements of the particular cell type being cultured. Serum that has been heat-inactivated (30 min to 1 hr at 56°C) is generally preferred, because heat treatment inactivates complement and is thought to reduce the number of contaminants. Serum is obtained frozen, then is thawed, divided into smaller portions, and refrozen until needed.

There is considerable lot-to-lot variation in FBS. Most suppliers will provide a sample of a specific lot and reserve a supply of that lot while the serum is tested for its suitability. The suitability of a serum lot depends upon the use. Frequently the ability of serum to promote cell growth equivalent to a laboratory standard is used to evaluate a serum lot. Once an acceptable lot is identified, enough of that lot should be purchased to meet the culture needs of the laboratory for an extended period of time.

Commercially prepared media containing L-glutamine are available, but many laboratories choose to obtain medium without L-glutamine, and then add it to a final concentration of 2 mM just before use. L-glutamine is an unstable amino acid that, upon storage, converts to a form cells cannot use. Breakdown of L-glutamine is temperature- and pH-dependent. At 4°C, 80% of the L-glutamine remains after 3 weeks, but at near incubator temperature (35°C) only half remains after 9 days (Barch et al., 1991). To prevent degradation, 100× L-glutamine should be stored frozen in aliquots until needed.

As well as practicing good aseptic technique, most laboratories add antimicrobial agents to medium to further reduce the risk of contamination. A combination of penicillin and streptomycin is the most commonly used antibiotic additive; kanamycin and gentamycin are used alone. Mycostatin and amphotericin B are the most commonly used fungicides (Rooney and Czepulkowski, 1992). Table A.3C.1 lists the final concentrations for the most commonly used antibiotics and antimycotics. Combining antibiotics in tissue culture media can be tricky, as some antibiotics are not compatible, and one may inhibit the action of another. Furthermore, combined antibiotics may be cytotoxic at lower concentrations than is true for the individual antibiotics. In addition, prolonged use of antibiotics may cause cell lines to develop antibiotic resistance. For this reason, some laboratories add antibiotics and/or fungicides to medium when initially establishing a culture but eliminate them from medium used in later subcultures.

All tissue culture medium, whether commercially prepared or prepared within the laboratory, should be tested for sterility prior to use. A small aliquot from each lot of medium is incubated 48 hr at 37°C and monitored for evidence of contamination such as turbidity (infected medium will be cloudy) and color change (if phenol red is the indicator, infected medium will turn yellow). Any contaminated medium should be discarded.

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TRYPSINIZING AND SUBCULTURING CELLS FROM A MONOLAYER

BASIC PROTOCOL

A primary culture is grown to confluency in a 60-mm petri dish or 25-cm^2 tissue culture flask containing 5 ml tissue culture medium. Cells are dispersed by trypsin treatment and then reseeded into secondary cultures. The process of removing cells from the primary culture and transferring them to secondary cultures constitutes a passage or subculture.

Materials

Primary cultures of cells
HBSS *without* Ca^{2+} and Mg^{2+} (see recipe), 37°C
0.25% (w/v) trypsin/0.2% EDTA solution (see recipe), 37°C
Complete medium with serum: e.g., DMEM supplemented with 10% to 15% (v/v) fetal bovine serum (complete DMEM-10; see recipe), 37°C

Sterile Pasteur pipets
37°C warming tray *or* incubator
Tissue culture plasticware or glassware including pipets and 25-cm^2 flasks or 60-mm petri dishes, sterile

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

1. Remove all medium from primary culture with a sterile Pasteur pipet. Wash adhering cell monolayer once or twice with a small volume of 37°C HBSS without Ca^{2+} and Mg^{2+} to remove any residual FBS, which may inhibit the action of trypsin.

   Use a buffered salt solution that is Ca^{2+}- and Mg^{2+}-free to wash cells. Ca^{2+} and Mg^{2+} in the salt solution can cause cells to stick together.

   If this is the first medium change, rather than discarding medium that is removed from primary culture, put it into a fresh dish or flask. The medium contains unattached cells that may attach and grow, thereby providing a backup culture.

2. Add enough 37°C trypsin/EDTA solution to culture to cover adhering cell layer.

3. Place plate on a 37°C warming tray 1 to 2 min. Tap bottom of plate on the countertop to dislodge cells. Check culture with an inverted microscope to be sure that cells are rounded up and detached from the surface.

   If cells are not sufficiently detached, return plate to warming tray for an additional minute or two.

4. Add 2 ml of 37°C complete medium. Draw cell suspension into a Pasteur pipet and rinse cell layer two or three times to dissociate cells and to dislodge any remaining adherent cells. As soon as cells are detached, add serum or medium containing serum to inhibit further trypsin activity that might damage cells.

   If cultures are to be split 1/3 or 1/4 rather than 1/2, add sufficient medium such that 1 ml of cell suspension can be transferred into each fresh culture vessel.

5. Add an equal volume of cell suspension to fresh dishes or flasks that have been appropriately labeled.

   Alternatively, cells can be counted using a hemacytometer (see Support Protocol 3) or Coulter counter and diluted to the desired density so a specific number of cells can be added to each culture vessel. A final concentration of ~5×10^4 cells/ml is appropriate for most subcultures.

   For primary cultures and early subcultures, 60-mm petri dishes or 25-cm^2 flasks are generally used; larger petri dishes or flasks (e.g., 150-mm dishes or 75-cm^2 flasks) may be used for later subcultures.

   Cultures should be labeled with patient name, lab number, date of subculture, and passage number.

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6. Add 4 ml fresh medium to each new culture. Incubate in a humidified 37°C, 5% CO_2 incubator.

   If using 75-cm^2 culture flasks, add 9 ml medium per flask.

   Some labs now use incubators with 5% CO_2 and 4% O_2. The low oxygen concentration is thought to simulate the in vivo environment of cells and to enhance cell growth.

7. If necessary, feed subconfluent cultures after 3 or 4 days by removing old medium and adding fresh 37°C medium.

8. Passage secondary culture when it becomes confluent by repeating steps 1 to 7, and continue to passage as necessary.

SUPPORT PROTOCOL 1

FREEZING HUMAN CELLS GROWN IN MONOLAYER CULTURES

It is sometimes desirable to store cell lines for future study. To preserve cells, avoid senescence, reduce the risk of contamination, and minimize effects of genetic drift, cell lines may be frozen for long-term storage. Without the use of a cryoprotective agent freezing would be lethal to the cells in most cases. Generally, a cryoprotective agent such as dimethylsulfoxide (DMSO) is used in conjunction with complete medium for preserving cells at –70°C or lower. DMSO acts to reduce the freezing point and allows a slower cooling rate. Gradual freezing reduces the risk of ice crystal formation and cell damage.

Materials

Log-phase monolayer culture of cells in petri dish
Complete medium
Freezing medium: complete medium (e.g., DMEM or RPMI; see recipes) supplemented with 10% to 20% (v/v) FBS and 5% to 10% (v/v) DMSO, 4°C
Benchtop clinical centrifuge (e.g., Fisher Centrific or Clay Adams Dynac) with 45°C fixed-angle or swinging-bucket rotor

1. Trypsinize log-phase monolayer culture of cells from plate (see Basic Protocol, steps 1 to 4).

   It is best to use cells in log-phase growth for cryopreservation.

2. Transfer cell suspension to a sterile centrifuge tube and add 2 ml complete medium with serum. Centrifuge 5 min at 300 to 350 × *g* (~1500 rpm in Fisher Centrific rotor), room temperature.

   Cells from three or more dishes from the same subculture of the same patient can be combined in one tube.

3. Remove supernatant and add 1 ml of 4°C freezing medium. Resuspend pellet.

4. Add 4 ml of 4°C freezing medium, mix cells thoroughly, and place on wet ice.

5. Count cells using a hemacytometer (see Support Protocol 3). Dilute with more freezing medium as necessary to get a final cell concentration of 10^6 or 10^7 cells/ml.

   To freeze cells from a nearly confluent 25-cm^2 flask, resuspend in roughly 3 ml freezing medium.

6. Pipet 1-ml aliquots of cell suspension into labeled 2-ml cryovials. Tighten caps on vials.

7. Place vials 1 hr to overnight in a –70°C freezer, then transfer to liquid nitrogen storage freezer.

   Alternatively, freeze cells in a freezing chamber in the neck of a Dewar flask according to manufacturer's instructions. Some laboratories place vials directly into the liquid nitrogen

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freezer, omitting the gradual temperature drop. Although this is contrary to the general recommendation to gradually reduce the temperature, laboratories that routinely use a direct-freezing technique report no loss of cell viability on recovery.

Keep accurate records of the identity and location of cells stored in liquid nitrogen freezers. Cells may be stored for many years and proper information is imperative for locating a particular line for future use.

FREEZING CELLS GROWN IN SUSPENSION CULTURE

ALTERNATE PROTOCOL

Freezing cells from suspension culture is similar in principle to freezing cells from monolayer. The major difference is that suspension cultures need not be trypsinized.

1. Transfer cell suspension to a centrifuge tube and centrifuge 10 min at 300 to 350 × *g* (~1500 rpm in Fisher Centrific centrifuge), room temperature.
2. Remove supernatant and resuspend pellet in 4°C freezing medium at a density of 10^6 to 10^7 cells/ml.

   Some laboratories freeze lymphoblastoid lines at the higher cell density because they plan to recover them in a larger volume of medium and because there may be a greater loss of cell viability upon recovery as compared to other types of cells (e.g., fibroblasts).
3. Transfer 1-ml aliquots of cell suspension into labeled cryovials and freeze as for monolayer cultures.

THAWING AND RECOVERING HUMAN CELLS

SUPPORT PROTOCOL 2

When cryopreserved cells are needed for study, they should be thawed rapidly and plated at high density to optimize recovery.

CAUTION: Protective clothing, particularly insulated gloves and goggles, should be worn when removing frozen vials or ampules from the liquid nitrogen freezer. The room containing the liquid nitrogen freezer should be well-ventilated. Care should be taken not to spill liquid nitrogen on the skin.

Materials

Cryopreserved cells stored in liquid nitrogen freezer
70% (v/v) ethanol
Complete medium (e.g., DMEM or RPMI; see recipes) containing 20% FBS (see recipe), 37°C
Tissue culture dish or flask

NOTE: All incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

1. Remove vial from liquid nitrogen freezer and immediately place it into a 37°C water bath. Agitate vial continuously until medium is thawed.

   The medium usually thaws in <60 sec.

   Cells should be thawed as quickly as possible to prevent formation of ice crystals which can cause cell lysis. Try to avoid getting water around the cap of the vial.
2. Wipe top of vial with 70% ethanol before opening.

   Some labs prefer to submerge the vial in 70% ethanol and air dry before opening.
3. Transfer thawed cell suspension into a sterile centrifuge tube containing 2 ml warm complete medium containing 20% FBS. Centrifuge 10 min at 150 to 200 × *g* (~1000 rpm in Fisher Centrific), room temperature. Discard supernatant.

   Cells are washed with fresh medium to remove residual DMSO.

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4. Gently resuspend cell pellet in small amount (~1 ml) of complete medium/20% FBS and transfer to properly labeled culture dish or flask containing the appropriate amount of medium.

   Cultures are reestablished at a higher cell density than that used for original cultures because there is some cell death associated with freezing. Generally, 1 ml cell suspension is reseeded in 5 to 20 ml medium.

5. Check cultures after ~24 hr to ensure that cells have attached to the plate.

6. Change medium after 5 to 7 days or when pH indicator (e.g., phenol red) in medium changes color. Keep cultures in medium with 20% FBS until cell line is reestablished.

   If recovery rate is extremely low, only a subpopulation of the original culture may be growing; be extra careful of this when working with cell lines known to be mosaic.

SUPPORT PROTOCOL 3

DETERMINING CELL NUMBER AND VIABILITY WITH A HEMACYTOMETER AND TRYPAN BLUE STAINING

Determining the number of cells in culture is important in standardization of culture conditions and in performing accurate quantitation experiments. A hemacytometer is a thick glass slide with a central area designed as a counting chamber.

The exact design of the hemacytometer may vary; the one described here is the Improved Neubauer from Baxter Scientific (Fig. A.3C.1). The central portion of the slide is the counting platform, which is bordered by a 1-mm groove. The central platform is divided into two counting chambers by a transverse groove. Each counting chamber consists of a silver footplate on which is etched a 3 × 3–mm grid. This grid is divided into nine secondary squares, each 1 × 1 mm. The four corner squares and the central square are used for determining the cell count. The corner squares are further divided into 16 tertiary squares and the central square into 25 tertiary squares to aid in cell counting.

Accompanying the hemacytometer slide is a thick, even-surfaced coverslip. Ordinary coverslips may have uneven surfaces, which can introduce errors in cell counting; therefore, it is imperative that the coverslip provided with the hemacytometer be used in determining cell number.

Cell suspension is applied to a defined area and counted so cell density can be calculated.

Materials

70% (v/v) ethanol
Cell suspension
0.4% (w/v) trypan blue *or* 0.4% (w/v) nigrosin, prepared in HBSS (see recipe)

Hemacytometer with coverslip (Improved Neubauer, Baxter Scientific)
Hand-held counter

Prepare hemacytometer

1. Clean surface of hemacytometer slide and coverslip with 70% alcohol.

   Coverslip and slide should be clean, dry, and free from lint, fingerprints, and watermarks.

2. Wet edge of coverslip slightly with tap water and press over grooves on hemacytometer. The coverslip should rest evenly over the silver counting area.

Prepare cell suspension

3. For cells grown in monolayer cultures, detach cells from surface of dish using trypsin (see Basic Protocol).

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4. Dilute cells as needed to obtain a uniform suspension. Disperse any clumps.

When using the hemacytometer, a maximum cell count of 20 to 50 cells per 1-mm square is recommended.

Load hemacytometer

5. Use a sterile Pasteur pipet to transfer cell suspension to edge of hemacytometer counting chamber. Hold tip of pipet under the coverslip and dispense one drop of suspension.

Suspension will be drawn under the coverslip by capillary action.

Hemacytometer should be considered nonsterile. If cell suspension is to be used for cultures, do not reuse the pipet and do not return any excess cell suspension in the pipet to the original suspension.

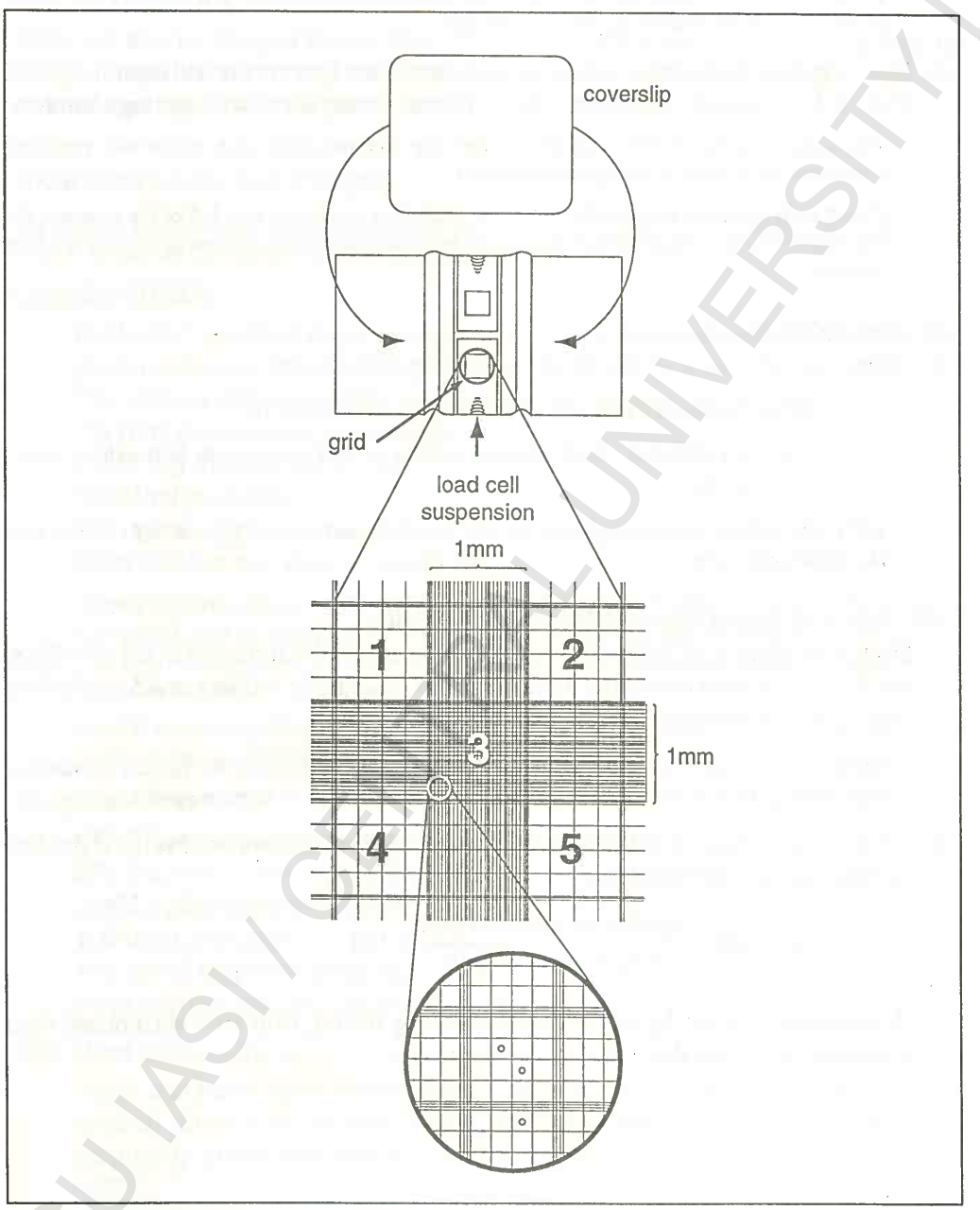

Figure A.3C.1 Hemacytometer slide (Improved Neubauer) and coverslip. Coverslip is applied to slide and cell suspension is added to counting chamber using a Pasteur pipet. Each counting chamber has a 3 × 3–mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted on each side of the hemacytometer (numbers added).

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6. Fill second counting chamber.

Count cells

7. Allow cells to settle for a few minutes before beginning to count. Blot off excess liquid.

8. View slide on microscope with 100× magnification.

   A 10× ocular with a 10× objective = 100× magnification.

   Position slide to view the large central area of the grid (section 3 in Fig. A.3C.1); this area is bordered by a set of three parallel lines. The central area of the grid should almost fill the microscope field. Subdivisions within the large central area are also bordered by three parallel lines and each subdivision is divided into sixteen smaller squares by single lines. Cells within this area should be evenly distributed without clumping. If cells are not evenly distributed, wash and reload hemacytometer.

9. Use a hand-held counter to count cells in each of the four corner and central squares (Fig. A.3C.1, squares numbered 1 to 5). Repeat counts for other counting chamber.

   Five squares (four corner and one center) are counted from each of the two counting chambers for a total of ten squares counted.

   Count cells touching the middle line of the triple line on the top and left of the squares. Do not count cells touching the middle line of the triple lines on the bottom or right side of the square.

Calculate cell number

10. Determine cells per milliliter by the following calculations:

    cells/ml = average count per square × dilution factor × 10^4

    total cells = cells/ml × total original volume of cell suspension from which sample was taken.

    10^4 is the volume correction factor for the hemacytometer: each square is 1 × 1 mm and the depth is 0.1 mm.

Stain cells with trypan blue to determine cell viability

11. Determine number of viable cells by adding 0.5 ml of 0.4% trypan blue, 0.3 ml HBSS, and 0.1 ml cell suspension to a small tube. Mix thoroughly and let stand 5 min before loading hemacytometer.

    Either 0.4% trypan blue or 0.4% nigrosin can be used to determine the viable cell number. Nonviable cells will take up the dye, whereas live cells will be impermeable to it.

12. Count total number of cells and total number of viable (unstained) cells. Calculate percent viable cells as follows:

$$\% \text{ viable cells} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

13. Decontaminate coverslip and hemacytometer by rinsing with 70% ethanol and then deionized water. Air dry and store for future use.

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PREPARING CELLS FOR TRANSPORT

Both monolayer and suspension cultures can easily be shipped in 25-cm^2 tissue culture flasks. Cells are grown to near confluency in a monolayer or to desired density in suspension. Medium is removed from monolayer cultures and the flask is filled with fresh medium. Fresh medium is added to suspension cultures to fill the flask. *It is essential that the flasks be completely filled with medium to protect cells from drying if flasks are inverted during transport.* The cap is tightened and taped securely in place. The flask is sealed in a leak-proof plastic bag or other leak-proof container designed to prevent spillage in the event that the flask should become damaged. The primary container is then placed in a secondary insulated container to protect it from extreme temperatures during transport. A biohazard label is affixed to the outside of the package. Generally, cultures are transported by same-day or overnight courier.

Cells can also be shipped frozen. The vial containing frozen cells is removed from the liquid nitrogen freezer and placed immediately on dry ice in an insulated container to prevent thawing during transport.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of cell buffers. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Complete DMEM

Dulbecco's modified Eagle medium, high-glucose formulation (e.g., Life Technologies), containing:
5%, 10%, or 20% (v/v) FBS, heat-inactivated (optional; see recipe)
1% (v/v) nonessential amino acids
2 mM L-glutamine (see recipe)
100 U/ml penicillin
100 µg/ml streptomycin sulfate
Filter sterilize and store ≤1 month at 4°C

Throughout this manual, the percentage of serum (usually fetal bovine serum, FBS) used in a protocol step is indicated by a numeral hyphenated to the base medium name. Thus, "complete DMEM-10" indicates that 10% FBS is used. Absence of a numeral indicates that no serum is used.

DMEM containing 4500 mg/liter D-glucose can be obtained from Life Technologies. DMEM is also known as Dulbecco's minimum essential medium.

Complete RPMI

RPMI 1640 medium (e.g., Life Technologies) containing:
2%, 5%, 10%, 15%, or 20% FBS, heat-inactivated (optional; see recipe)
2 mM L-glutamine (see recipe)
100 U/ml penicillin
100 µg/ml streptomycin sulfate
Filter sterilize and store ≤1 month at 4°C

FBS (fetal bovine serum)

Thaw purchased fetal bovine serum (shipped on dry ice and kept frozen until needed). Store 3 to 4 weeks at 4°C. If FBS is not to be used within this time, aseptically divide into smaller aliquots and refreeze until used. Store ≤1 year at –20°C.

Repeated thawing and refreezing should be avoided as it may cause denaturation of the serum.

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Inactivated FBS (FBS that has been treated with heat to inactivate complement protein and thus prevent an immunological reaction against cultured cells) is useful for a variety of purposes. It can be purchased commercially or made in the lab. To inactivate FBS, heat serum 30 min to 1 hr in a 56°C water bath.

Alternatively, FBS may be inactivated through radiation treatment.

L-glutamine, 0.2 M (100×)

Thaw commercially prepared frozen L-glutamine or prepare an 0.2 M solution in water, aliquot aseptically into usable portions, then refreeze. For convenience, L-glutamine can be stored in 1-ml aliquots if 100-ml bottles of medium are used and in 5-ml aliquots if 500-ml bottles are used. Store ≤1 year at –20°C.

Many laboratories supplement medium with 2 mM L-glutamine—1% (v/v) of 100× stock—just prior to use.

HBSS (Hanks balanced salt solution)

5.4 mM KCl
0.3 mM Na_2HPO_4
0.4 mM KH_2HPO_4
4.2 mM $NaHCO_3$
1.3 mM $CaCl_2$
0.5 mM $MgCl_2$
0.6 mM $MgSO_4$
137 mM NaCl
5.6 mM D-glucose
0.02% (w/v) phenol red (optional)
Add H_2O to 1 liter and adjust pH to 7.4

HBSS can be purchased from Biofluids or Whittaker.

To prepare HBSS *without* Ca^{2+} and Mg^{2+}, omit the $CaCl_2$, $MgCl_2$, and $MgSO_4$.

Trypsin/EDTA solution

Prepare in sterile HBSS (see recipe) or 0.9% (w/v) NaCl:
0.25% (w/v) trypsin
0.2% (w/v) EDTA
Store ≤1 year (until needed) at –20°C

Specific applications may require different concentrations of trypsin.

Trypsin/EDTA solution is commercially available in various concentrations including 10×, 1×, and 0.25% (w/v). It is received frozen from the manufacturer and can be thawed and aseptically divided into smaller volumes. Preparing trypsin/EDTA from powdered stocks may reduce its cost; however, most laboratories prefer commercially prepared solutions for convenience.

EDTA (disodium ethylenediamine tetraacetic acid) is added as a chelating agent to bind Ca^{2+} and Mg^{2+} ions that can interfere with the action of trypsin.

COMMENTARY

Background Information

At its inception in the early twentieth century, tissue culture was applied to the study of tissue fragments in culture. New growth in culture was limited to cells that migrated out from the initial tissue fragment. Tissue culture techniques evolved rapidly, and since the 1950s culture methods have allowed the growth and study of dispersed cells in culture (Freshney, 1993). Cells dispersed from the original tissue can be grown in monolayers and passaged repeatedly to give rise to a relatively stable cell line.

Four distinct growth stages have been described for primary cells maintained in culture. First, cells adapt to the in vitro environment. Second, cells undergo an exponential growth phase lasting through ~30 passages. Third, the

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growth rate of cells slows, leading to a progressively longer generation time. Finally, after 40 or 50 passages, cells begin to senesce and die (Lee, 1991). It may be desirable to study a particular cell line over several months or years, so monolayer cultures can be preserved to retain the integrity of the cell line. Aliquots of early passage cell suspensions are frozen, then thawed, and cultures reestablished as needed. Freezing monolayer cultures prevents changes due to genetic drift and avoids loss of cultures due to senescence or accidental contamination (Freshney, 1993).

Cell lines are commercially available from a number of sources, including the American Type Culture Collection (ATCC) and the Human Genetic Mutant Cell Repository at the Coriell Cell Repository (CCR). These cell repositories are a valuable resource for researchers who do not have access to suitable patient populations.

Critical Parameters

Use of aseptic technique is essential for successful tissue culture. Cell cultures can be contaminated at any time during handling, so precautions must be taken to minimize the chance of contamination. All supplies and reagents that come into contact with cultures must be sterile and all work surfaces should be kept clean and free from clutter.

Cultures should be 75% to 100% confluent when selected for subculture. Growth in monolayer cultures will be adversely affected if cells are allowed to become overgrown. Passaging cells too early will result in a longer lag time before subcultures are established. Following dissociation of the monolayer by trypsinization, serum or medium containing serum should be added to the cell suspension to stop further action by trypsin that might be harmful to cells.

When subculturing cells, add a sufficient number of cells to give a final concentration of ~5×10^4 cells/ml in each new culture. Cells plated at too low a density may be inhibited or delayed in entry into growth stage. Cells plated at too high a density may reach confluence before the next scheduled subculturing; this could lead to cell loss and/or cessation of proliferation. The growth characteristics for different cell lines vary. A lower cell concentration (10^4 cells/ml) may be used to initiate subcultures of rapidly growing cells, and a higher cell concentration (10^5 cells/ml) may be used to initiate subcultures of more slowly growing cells. Adjusting the initial cell concentration permits establishment of a regular, convenient schedule for subculturing—e.g., once or twice a week (Freshney, 1993).

Cells in culture will undergo changes in growth, morphology, and genetic characteristics over time. Such changes can adversely affect reproducibility of laboratory results. Nontransformed cells will undergo senescence and eventual death if passaged indefinitely. The time of senescence will vary with cell line, but generally at between 40 and 50 population doublings fibroblast cell lines begin to senesce. Cryopreservation of cell lines will protect against these adverse changes and will avoid potential contamination.

Cultures selected for cryopreservation should be in log-phase growth and free from contamination. Cells should be frozen at a concentration of 10^6 to 10^7 cells/ml. Cells should be frozen gradually and thawed rapidly to prevent formation of ice crystals that may cause cells to lyse. Cell lines can be thawed and recovered after long-term storage in liquid nitrogen. The top of the freezing vial should be cleaned with 70% alcohol before opening to prevent introduction of contaminants. To aid in recovery of cultures, thawed cells should be reseeded at a higher concentration than that used for initiating primary cultures. Careful records regarding identity and characteristics of frozen cells as well as their location in the freezer should be maintained to allow for easy retrieval.

For accurate cell counting, the hemacytometer slide should be clean, dry, and free from lint, scratches, fingerprints, and watermarks. The coverslip supplied with the hemacytometer should always be used because it has an even surface and is specially designed for use with the counting chamber. Use of an ordinary coverslip may introduce errors in cell counting. If the cell suspension is too dense or the cells are clumped, inaccurate counts will be obtained. If the cell suspension is not evenly distributed over the counting chamber, the hemacytometer should be washed and reloaded.

Anticipated Results

Confluent cell lines can be successfully subcultured in the vast majority of cases. The yield of cells derived from monolayer culture is directly dependent on the available surface area of the culture vessel (Freshney, 1993). Overly confluent cultures or senescent cells may be difficult to trypsinize, but increasing the time of trypsin exposure will help dissociate resistant cells. Cell lines can be propagated to get

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sufficient cell populations for cytogenetic, biochemical, and molecular analyses.

It is well accepted that anyone can successfully freeze cultured cells; it is thawing and recovering the cultures that presents the problem. Cultures that are healthy and free from contamination can be frozen and stored indefinitely. Cells stored in liquid nitrogen can be successfully thawed and recovered in over 95% of cases. Several aliquots of each cell line should be stored to increase the chance of recovery. Cells should be frozen gradually, with a temperature drop of ~1°C per minute, but thawed rapidly. Gradual freezing and rapid thawing prevents formation of ice crystals that might cause cell lysis.

Accurate cell counts can be obtained using the hemacytometer if cells are evenly dispersed in suspension and free from clumps. Determining the proportion of viable cells in a population will aid in standardization of experimental conditions.

Time Considerations

Establishment and maintenance of mammalian cell cultures require a regular routine for preparing media and feeding and passaging cells. Cultures should be inspected regularly for signs of contamination and to determine if the culture needs feeding or passaging.

Literature Cited

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Rooney, D.E. and Czepulkowski, B.H. (eds.) 1992. Human Cytogenetics: A Practical Approach, Vol. I. Constitutional Analysis, 2nd ed. IRL Press, Washington, D.C.

Westinghouse Electric Company. 1976. Westinghouse sterilamp germicidal ultraviolet tubes. Westinghouse Electric Corp., Bloomfield, NJ.

Key Reference

Lee, E.C. 1991. See above.

Contains pertinent information on cell culture requirements including medium preparation and sterility. Also discusses trypsinization, freezing and thawing, and cell counting.

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Importing Biological Materials

Scientific cooperation is no longer confined to laboratories at the same institution, nor between laboratories within the boundaries of the United States. More than ever, scientists are forming collaborations with their peers outside the U.S., leading not only to the exchange of knowledge and ideas, but also to the exchange of biological reagents. Thus, investigators need be aware that an import permit may be required to obtain purified proteins, chemically synthesized materials, cell lines, tissues, and other biological materials from abroad. Before requesting any biological material from a laboratory outside the U.S., it is the responsibility of the "receiver" to ascertain whether an import permit is required, to apply for the permit if one is determined to be necessary, and to provide the "shipper" with an official copy of the permit to be included with the shipped material.

There are two government agencies in place to issue permits for the importation of most biologicals. Materials derived from all animals are subject to regulations set forth by the United States Department of Agriculture (USDA) and must be cleared by USDA inspectors at the port of arrival before entry into the United States is authorized. In general, an import permit issued by the USDA is necessary for receipt of any animal-derived material or any biological material (including proteins) that has been in contact with materials of animal origin. The USDA does not have regulatory authority over the importation of human or nonhuman primate material unless it is produced via tissue culture, due to the use of bovine material (fetal bovine serum or calf serum) in tissue culture. The U.S. Public Health Service (UPHS) has jurisdiction over human and nonhuman primate materials. According to UPHS regulations, any etiologic agent, animal host, or vector of human disease may not be imported in the United States nor distributed after importation without being accompanied by a permit issued by the Director of the Centers for Disease Control. Failure to obtain the appropriate import permit will result in the shipment being held at the port of entry until a permit is applied for and issued (a 4- to 6-week process), or it may result in shipment confiscation and destruction by the quarantine officer present.

IMPORTING RESTRICTED BIOLOGICAL MATERIALS

The following is a list of restricted biological materials requiring an import permit: proteins, monoclonal and polyclonal antibodies, antisera, immunoglobins, recombinant products, enzymes, hormones, immunoassay components or kits, animal tissues, blood, cells, cell lines, RNA/DNA extracts, plasmids, vectors, microorganisms—e.g., fungi, bacteria, viruses—and other products that are derived from or have been in contact with animal-derived materials. In addition, materials received from countries shown not to be free of foot-and-mouth disease (see Title 9, code of Federal Regulations, Part 94.1) require an import permit. Note that the USDA will not permit the importation of cell cultures, monoclonal antibodies, ascites fluid, or bovine serum from countries where rinderpest and foot-and-mouth disease are present unless the imported materials are determined to be virus-free. Cell cultures being imported into the U.S. must have been grown only with fetal bovine serum of U.S., Canadian, New Zealand, or Australian origin. Material exposed to bovine products originating in the United Kingdom, Switzerland, Ireland, France, or Portugal may NOT be used, either directly or indirectly, in any animals due to the presence of bovine spongiform encephalopathy (BSE) in those countries, and the material is restricted to in vitro uses only.

CRITERIA FOR HANDLING IMPORTED RESTRICTED MATERIALS

An import permit is only valid for transfer of the material(s) listed on the permit (a permit can cover more than one item), and for shipment between the two laboratories listed on the permit. Therefore, for each laboratory abroad from which biological materials are to be imported, a separate import permit is required. Once biological materials have been received from abroad, the import permit still imposes regulations on how the materials are handled by the receiver. Import permits do not usually authorize direct or indirect exposure of domestic animals—work is limited to in vitro uses only. In addition, permits are only valid for work conducted or directed by the permit holder at his or her facilities. Materials cannot be removed to another location, nor distributed to others, without USDA authorization. There

Contributed by Paula Wolf

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is one exception: monoclonal antibodies produced by hybridoma cell lines may be commercially distributed for in vitro uses.

IMPORTING NONRESTRICTED BIOLOGICAL MATERIALS

Any of the restricted materials that do not contain animal- or cell culture-derived products or additives such as albumin, serum, or gelatin, or were not exposed to any infectious agents of agricultural concern, do not need an official import permit. However, an accompanying declaration is required with each shipment to indicate that the material does not contain any animal- or cell culture-derived products or additives. This information should be supplied with each shipment in a clear and concise manner, and be available for the USDA Port of Entry Inspector's review. A separate memo or letter should be included with the shipping documents, such as U.S. Customs declaration and invoice. Note, whether a particular biological material requires an import permit cannot be decided by the investigator; the investigator must contact the USDA for a decision well in advance of making arrangements for importing the material.

PACKAGING BIOLOGICAL MATERIALS FOR IMPORTATION

Imported biological materials are subject to packaging and shipping requirements of various federal and international regulations. Proper packaging is the primary consideration and of utmost importance in the safe transportation of hazardous materials. If the biological material being shipped is infectious or radioactive, this information has to be noted on the outside of the package. In addition, if dry ice is used for shipment, that must be clearly stated on the package. It is of course advantageous to the investigator that the shipped material be packaged in the most efficient way possible, as well as in a form which will ensure its stability. If proteins are to be shipped, it may be better to send the samples lyophilized or blotted on a membrane, rather than in liquid form. This will omit the need for dry ice or ice packs in the packaging, which will save on shipping costs as well as ensure the samples will be stable if receipt of the package meets with unforeseen delays. If sending materials that require dry ice or ice packs, such as antibodies or antisera, there must be enough dry ice to allow the package to maintain the appropriate temperature during shipping for approximately 5 days. This requires utilizing much larger packaging boxes than are normally used to ship the same material within the United States. When shipping cell lines, sometimes it is better to send cells in culture, rather than frozen aliquots. To send cells in culture, the tissue culture flask containing the live cells must be filled to the very top with the appropriate medium, tightly capped, and the seal secured with parafilm. If the tissue culture flask is not completely filled with medium, the resulting air pockets will cause the flasks to explode in flight. Thus, the packaging of any biological material for shipping is an import consideration to ensure success of the scientific collaboration!

OBTAINING AN IMPORT PERMIT APPLICATION

The Division of Veterinary Services of the USDA Animal and Plant Health Inspection Service (APHIS) administers regulatory programs to control the import/export of biological materials. Import permits can be obtained from either local USDA port offices or from APHIS directly: USDA, APHIS, VETERINARY SERVICES, National Center for Import-Export Products Program, 4700 River Road Unit 40, Riverdale, MD 20737-1231, Tel: (301) 734-7830, Fax: (301) 734-8226.

When submitting an import application, (1) obtain the import permit well in advance of the proposed shipping date; (2) inform the supplier of the USDA's requirements, and do not allow shipment of materials until an import permit has been issued and the supplier has received a copy of the permit; (3) list all the potential U.S. ports of entry; (4) specify whether the material is for in vitro or in vivo use and whether it is for commercial distribution or for research in one's own laboratory; (5) list any treatments the material has undergone, such as processing and purification steps involving pH, heat, chromatography, or other methods; and (6) indicate the source(s) of nutrient factors in culture media, cell line designation and history, whether enzymes were used, and what types of viruses (if any) are being studied in the laboratory of origin (all must be listed).

RESOURCES

APHIS Web Site

More information regarding APHIS programs and import/export can be obtained on their internet home page at http://www.aphis.usda.gov.

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Fax Service of Veterinary Services, National Center for Import-Export

By calling (301) 734-4952, the investigator can access an automated document retrieval system and arrange to have an import application faxed directly, and choose from a menu of documents that describe general instructions for completing the application, as well as information regarding the importation of biological materials from 18 distinct categories.

Contributed by Paula Wolf
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Cambridge, Massachusetts

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SELECTED SUPPLIERS OF REAGENTS AND EQUIPMENT

Listed below are addresses and phone numbers of commercial suppliers who have been recommended for particular items used in our manuals because: (1) the particular brand has actually been found to be of superior quality, or (2) the item is difficult to find in the marketplace. Consequently, this compilation may not include some important vendors of biological supplies. For comprehensive listings, see *Linscott's Directory of Immunological and Biological Reagents* (Santa Rosa, CA), *The Biotechnology Directory 1998* (Stockton Press, New York), the annual Buyers' Guide supplement to the journal *Bio/Technology*, as well as various sites on the Internet.

A.J. Buck
11407 Cronhill Drive
Owings Mill, MD 21117
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(410) 581-1800

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S.D.R. Clinical Technology
213 Eastern Valley Way
Middle Cove N.S.W.
2068 Australia
(61) 02-9958-2688
FAX:(61) 02-9958-2655

Abbott Laboratories
Diagnostics Division, Dept. 90H
Abbott Park, IL 60064
(800) 323-9100 FAX:(847) 937-1511
(847) 937-6100

ABI Biotechnologies
See Perkin-Elmer

ABI Biotechnology
40 Scurfield Boulevard
Winnipeg, Manitoba
R3Y 164 Canada
(204) 488-4090 FAX:(204) 488-4063

Accurate Chemical and Scientific
300 Shames Drive
Westbury, NY 11590
(800) 645-6264 FAX: (516) 997-4948
(516) 333-2221

Ace Glass
1430 NW Boulevard
Vineland, NJ 08360
(800) 223-4524 FAX: (800) 543-6752
(609) 692-3333

Acros Organic
See Fisher Scientific

Action Scientific
P.O. Box 1369
Carolina Beach, NC 28428
(800) 678-1033 FAX: (910) 458-0407
(910) 458-0401

Adobe Systems
1585 Charleston Road
P.O. Box 7900
Mountain View, CA 94039
(800) 833-6687 FAX: (415) 961-3769
(415) 961-4400

Advanced Biotechnologies
9108 Guilford Road
Columbia, MD 21046
(800) 426-0764 FAX: (301) 497-9773
(301) 792-9779

Advanced Magnetics
See PerSeptive Biosystems

Advanced Process Supply
See Naz-Dar-KC Chicago

Advet
Industrivagen 24
S-972 54 Lulea, Sweden
(46) 0920-211887
FAX: (46) 0920-13773

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Girton, Cambridge
CB3 OJX United Kingdom
(0223) 277192 FAX: (0223) 277605

Agar Scientific
66a Cambridge Road
Stansted, CM24 8DA
United Kingdom
(44) 1279-81-3519
FAX: (44) 1279-81-5106

A/G Technology
101 Hampton Avenue
Needham, MA 02194
(800) AGT-2535 FAX: (617) 449-5786
(617) 449-5774

Agouron Pharmaceuticals
10350 N. Torrey Pines Road
La Jolla, CA 92037
(619) 622-3000 FAX: (619) 622-3298

Agracetus
8520 University Green
Middleton, WI 53562
(608) 836-7300 FAX: (608) 836-9710

AIDS Research and Reference Reagent Program
U.S. Department of Health and Human Services
685 Lofstrand Lane
Rockville, MD 20850
(301) 340-0245 FAX: (301) 340-9245

Air Products and Chemicals
7201 Hamilton Boulevard
Allentown, PA 18195
(800) 345-3148 FAX: (215) 481-4381
(610) 481-3864

ALA Scientific Instruments
1100 Shames Drive
Westbury, NY 11590
(516) 997-5780 FAX: (516) 997-0528

Aladin Enterprises
1255 23rd Avenue
San Francisco, CA 94122
(415) 468-0433 FAX: (415) 468-5607

Alcide
8561 154th Avenue NE
Redmond, WA 98052
(800) 543-2133 FAX: (206) 861-0173
(206) 882-2555

Aldrich Chemical
P.O. Box 2060
1001 West St. Paul Avenue
Milwaukee, WI 53233
(800) 558-9160 FAX: (800) 962-9591
(414) 273-3850 FAX: (414) 273-4979

Alexis
6181 Cornerstone Court East
P.O. Box 927190
San Diego, CA 92121
(800) 900-0065 FAX: (619) 658-9224
(619) 658-0065

Alfa Laval
333 Rue du Progres
1210 Brussels, Belgium
2-2442811

Alice King Chatham Medical Arts
11915-17 Inglewood Avenue
Hawthorne, CA 90250
(310) 970-1834 FAX: (310) 970-0121

Allelix
6850 Gorway Drive
Mississauga, Ontario
L4V 1P1 Canada
(416) 677-0831 FAX: (416) 677-9595

Alltech Associates
Applied Science Labs
2051 Waukegan Road
P.O. Box 23
Deerfield, IL 60015
(800) 255-8324 FAX: (847) 948-1078
(847) 948-8600

Alomone Labs
Shatner Center 3
P.O. Box 4287
Jerusalem 91042, Israel
(800) 618-1644 (US)
(800) 267-1342 (Canada)
(972) 2-652-8002
FAX: (972) 2-652-5233

Altec Plastics
116 B Street
Boston, MA 02127
(617) 269-1400 FAX: (617) 269-8484

Alza
950 Page Mill Road
P.O. Box 10950
Palo Alto, CA 94303
(800) 692-2990 FAX: (415) 962-2488
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Amac
160B Larrabee Road
Westbrook, ME 04092
(800) 458-5060 FAX: (207) 854-0116
(207) 854-0426

Ambion
2130 Woodward Street, Suite 200
Austin, TX 78744
(800) 888-8804 FAX: (512) 445-7139
(512) 445-6979

American Association of Blood Banks
College of American Pathologists
325 Waukegan Road
Northfield, IL 60093
(800) 323-4040 FAX: (708) 446-8819
(708) 446-8800

American Bio-Technologies
359 Allston Street
Cambridge, MA 02139
(800) 542-2281 FAX: (617) 491-9015
(617) 547-5535

American Bioanalytical
10 Huron Drive
Natick, MA 01760
(800) 443-0600 FAX: (508) 655-2754
(508) 655-4336

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American Scientific Products
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American Society for Histocompatibility and Immunogenetics Cell Repository
P.O. Box 15804
Lenexa, KS 66285
(913) 541-0009 FAX: (913) 541-0156

American Type Culture Collection (ATCC)
12301 Parklawn Drive
Rockville, MD 20852
(800) 638-6597 FAX: (301) 231-5826
(301) 881-2600

Amersham
2636 South Clearbrook Drive
Arlington Heights, IL 60005
(800) 323-9750 FAX: (800) 228-8735
(708) 593-6300 FAX: (708) 593-8010

Amersham International
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Little Chalfont, Buckinghamshire
HP7 9NA United Kingdom
01494-54400

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Arlington Heights, IL 60004
(800) 633-4123 FAX: (800) 826-9863

Amersham Pharmacia Biotech
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P.O. Box 1327
Piscataway, NJ 08855
(800) 526-3593 FAX: (908) 457-0557
(908) 457-8000

Amgen Biologicals
Amgen Center
Thousand Oaks, CA 91320
(800) 282-6436 FAX: (805) 498-9377
(805) 499-5725

Amicon
Scientific Systems Division
72 Cherry Hill Drive
Beverly, MA 01915
(800) 426-4266 FAX: (508) 777-6204
(508) 777-3622

Amrad
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Kew, Victoria 3101, Australia
(03) 853-0022 FAX: (03) 853-0202

Amresco
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Solon, OH 44139
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(216) 349-1199

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(650) 494-3894

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(408) 452-5055

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Denver, CO 80231
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Anatomia
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London, NW1, United Kingdom
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FAX: (44) 1713-873916

Ancell
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(612) 439-0835

Anglian Biotec
Whitehall Road
Colchester, Essex
CO2 8HA United Kingdom
(44) 1206 866007
FAX: (44) 1206 860516

Animal Identification and Marking Systems (AIMS)
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Piscataway, NJ 08854
(908) 356-9366 FAX: (908) 271-8857

Ann Arbor Plastics
2289 S. State Road
Ann Arbor, MI 48104
(800) 783-3674 FAX: (313) 994-8622
(313) 994-3674

Apple Scientific
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Chesterland, OH 44026
(216) 729-3056 FAX: (216) 729-0928

Applied Biosystems
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(415) 570-6667

Applied Imaging
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Santa Clara, CA 95051
(800) 634-3622 FAX: (408) 562-0264
(408) 562-0250

Applied Immunosciences
5301 Patrick Henry Drive
Santa Clara, CA 95054
(408) 497-9200 FAX: (408) 980-5888

Applied Neuroscience
142 Gloucester Place
London, NW1 6DT United Kingdom
(44) 181-809-3947

Appligene Oncor
Parc d'Innovation
Rue Geiler de Kaysersberg, BP 72
67402 Illkirch, Cedex, France
(33) 0-3-8867-2267
FAX: (33) 0-3-8867-1945

Applikon
1165 Chess Drive, Suite G
Foster City, CA 94404
(650) 578-1396 FAX: (650) 578-8836

APV Gaulin
500 Research Drive
Wilmington, MA 01887
(508) 988-9300 FAX: (508) 988-9111

Aqualon
1313 N. Market Street
Wilmington, DE 19899
(800) 345-8104 FAX: (302) 594-6660
(302) 594-6600

Aquebogue Machine and Repair Shop
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Main Road
Aquebogue, NY 11931
(516) 722-3635 FAX: (516) 722-3106

Ariad Pharmaceuticals
26 Landsdowne Street
Cambridge, MA 02139
(617) 494-0400 FAX: (617) 494-8144

Armour Pharmaceuticals
500 Arcola Road
Collegeville, PA 19426
(800) 727-6737 FAX: (610) 454-8940
(610) 454-8975

Assymetrix
3380 Central Expressway
Santa Clara, CA 95051
(408) 731-5000 FAX: (408) 481-0422

AT Biochem
30 Spring Mill Drive
Malvern, PA 19355
(610) 889-9300 FAX: (610) 889-9304

ATC Diagnostics
31 New York Avenue
Framingham, MA 01701
(800) 553-7042 FAX: (508) 879-6462
(508) 935-1100

ATCC
See American Type Culture Collection

ATR
P.O. Box 460
Laurel, MD 20725
(800) 827-5931 FAX: (410) 792-2837
(301) 470-2799

Aurora Biosciences
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CHAPTER 5
Production of Recombinant Proteins

INTRODUCTION

This chapter deals with the production of recombinant proteins using the expression systems in common use. Methods describing the actual construction of the expression vectors or plasmids containing the genes coding the proteins of interest are described in detail elsewhere (Sambrook et al., 1989; Ausubel et al., 1997).

The most widely used and most important expression systems are those using *E. coli* as the host cell. An overview of the production of proteins in *E. coli* is provided in *UNIT 5.1*. General strategies for gene expression described include direct intracellular expression, secretion of the protein into the periplasmic space, and the expression of fusion proteins. Some of these strategies (e.g., secretion procedures) are used to overcome the common problem of inclusion body formation (see *UNIT 6.1* for additional discussion); others (e.g., fusions involving affinity tags) are used simply to assist in protein purification. Many of the expression vectors discussed are commercially available and suppliers are indicated throughout the text.

Protocols describing protein expression in transformed *E. coli* cells are provided in *UNITS 5.2 & 5.3*. First, protein expression in *E. coli* requires that a suitable host strain (tabulated in *UNIT 5.2*) be transformed with an expression vector. Electroporation, a quick and simple method for transforming *E. coli* host strains, is presented in *UNIT 5.2*. Second, the growth conditions and methods used to induce gene expression are optimized for the particular host strain and controllable transcriptional promoter used. Standard procedures for small-scale expression in shaker flasks using several of the most common expression vectors are also presented, along with protocols for determining plasmid stability and storing transformed strains at low temperature. Finally, methods for establishing the level of protein expression as well as solubility and location (intracellular or periplasmic) are described.

Once suitable host/vector combinations have been selected on the basis of the small-scale experiments described in *UNIT 5.2*, large-scale protein production can be carried out in fermentors with careful monitoring of cell growth. *UNIT 5.3* describes fermentation in batch mode using a fixed amount of medium. For higher-density fermentations, the fed-batch process is described: in this procedure the growth carbon source is continuously fed into the culture. Modifications to the standard fed-batch process are used to introduce the heavy atom derivative selenomethionine into recombinant proteins to facilitate X-ray structure determination and to uniformly label proteins with combinations of the stable isotopes ^{2}H, ^{13}C, and ^{15}N. Isotopically labeled proteins are required for high-resolution structural studies using nuclear magnetic resonance (NMR) spectroscopy (see *UNIT 7.1* for additional details). Protocols for growth monitoring and sterility checking are also included.

There are several eukaryotic cell–based expression systems available for overproducing recombinant proteins (consult Ausubel et al., 1997, Chapter 16), and most of these will eventually be covered in this chapter. One system, which uses insect cells infected with recombinant baculovirus, has become widely used. An overview of protein expression using the baculovirus system is given in *UNIT 5.4*. References covering the molecular

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biology required to construct the recombinant baculovirus vector that expresses the gene of interest are provided in the overview. Furthermore, references to protocols describing small-scale expression experiments are supplied in the introduction to *UNIT 5.5*.

The large-scale growth of insect cells infected with baculovirus is carried out in bioreactors, requiring large (liter) amounts of viral stock (*UNIT 5.5*). These stocks must have known titers and are analyzed in small-scale (1- to 3-liter) cultures in order to evaluate and optimize protein expression. For developing protein purification methods and for isolating moderate amounts of recombinant protein, small-scale cultures in shaker or spinner culture flasks may provide adequate starting material. Large-scale insect cell culture is carried out with more sophisticated equipment using bioreactors in either batch mode or perfusion mode. Cell harvesting is carried out by centrifugation or tangential-flow microfiltration. The cells (for intracellular expressed proteins) or the culture medium (for secreted proteins) are retained for downstream processing. Other protocols in this unit provide methods for preparing samples for SDS-PAGE to check protein expression levels and for monitoring nutrient levels in culture media.

Yeast is another important eukaryotic host used for protein expression. The molecular biology of *Saccharomyces cerevisiae* (baker's yeast) is described in Chapter 13 of Ausubel et al. (1997). An overview of protein expression in *S. cerevisiae* is given in *UNIT 5.6*, which details, as a case study, some of the machinations required to achieve high-level production of the human glycoprotein antithrombin 111 secreted into the extracellular medium.

Other strains of yeast apart from *S. cerevisiae* have also been used for heterologous protein expression, including the methylotropic yeast *Pichia pastoris*. There may be some advantages to using this host system over the more common *S. cerevisiae*, as expression levels, especially for intracellular proteins, can be higher. Protein expression using the *P. pastoris* system is reviewed in *UNIT 5.7*. The commonly used expression vectors are described, together with many examples of proteins successfully expressed with the system. Protocols describing the culture of the *S. cerevisiae* and *P. pastoris* strains with various expression vectors for heterologous protein expression are provided in *UNIT 5.8*.

An overview of protein expression using mammalian cells is presented in *UNIT 5.9*. The unit reviews the stages involved in protein production using the stable expression approach. As post-transcriptional modification and intracellular protein transportation are integral components of mammalian protein expression, descriptions of these mechanisms are included. Protocols for the production of recombinant proteins in mammalian cells using various culture configurations are described in *UNIT 5.10*. These are supplemented with a number of support protocols that include the first steps in downstream processing.

The transient expression of proteins in mammalian cells using a viral vector system is described in *UNITS 5.11-5.15*. Methods for constructing recombinant vaccinia virus are presented in *UNITS 5.11-5.13 & 5.15*. The characterization of the recombinant virus is described in *UNIT 5.14*. Proteins produced using this expression system are subject to normal mammalian post-translational processing.

The choice of protein expression system does depend on the intended use and characteristics of the protein. If a protein can be expressed in *E. coli* in a soluble state either directly into the cytoplasm or by secretion into the periplasm, then it may be possible to isolate large amounts of correctly folded and active protein (e.g., see *UNIT 6.2*). On the other hand, if the protein must be post-translationally modified (i.e., glycosylated or phosphorylated), a eukaryotic expression system must be used. Furthermore, if the protein is expressed in an insoluble state in *E. coli*, one way to circumvent this problem is to try expression in an eukaryotic system.

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The baculovirus system complements *E. coli* expression systems and for many investigators is the system of choice, especially for expressing post-translationally modified proteins. Yeast has the advantage that it is cheap to grow (analogous to *E. coli*) but can produce hyperglycosylated proteins; moreover, occasionally protein produced at high levels is insoluble (again analogous to *E. coli*). If a glycoprotein is required for clinical studies, it must usually be produced by mammalian cell expression, as discussed in detail in *UNITS 5.9 & 5.10*. However, no expression system is without its drawbacks; some of the problems reported using the baculovirus and yeast systems are mentioned in *UNITS 5.4 & 5.6-5.8* and elsewhere (Bradley, 1990). In addition, a brief summary of the advantages and disadvantages of all the commonly used host systems is given in the booklet *The Recombinant Protein Handbook* (Pharmacia Biotech, 1994).

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Table 5.9.1 Common Mammalian Cell Hosts

Cell line	Description	Growth	Utility	Source[a]
Human				
Namalwa	Burkitt's lymphoma–transformed lymphoblastoid cell	Large-scale suspension	Production of α interferon (e.g., by Burroughs Wellcome)	ATCC #CRL-1432
HeLa	Aneuploid cervical carcinoma cell	Small-scale suspension	Production of small quantities of research material (few mg)	ATCC #CCL-2
293	Transformed kidney cell	Small-scale suspension	Production of small quantities of research material (few mg)	ATCC #CCL-1573
WI-38	Human diploid normal embryonic lung cell	Attachment only	Host for virus production	ATCC #CCL-75
MRC-5	Human diploid normal embryonic lung cell	Attachment only	Hardier host for virus production—e.g., hepatitis A	ATCC #CCL-171
HepG2	Liver carcinoma transformed cell	Attachment	Small-scale evaluation of expression—e.g., HBVsAg	ATCC #HB-8065
Rodent				
3T3	Swiss mouse embryo fibroblast	Attachment	Used in testing transforming agents, expression evaluation	ATCC #CCL-92
L-929	Normal connective tissue fibroblasts	Attachment	Small-scale evaluations of expression	ATCC #CCL-1
Myeloma (e.g., NS/O)	Many types	Large-scale suspension	Monoclonal antibody production	ATCC (and commercial sources)
BHK-21	Baby hamster kidney cell	Large-scale suspension	Host for virus production or for stable gene integration	ATCC #CCL-10
CHO-K1	Chinese hamster ovary cell	Large-scale suspension	Used with glutamine synthetase system	ATCC #CCL-61
CHO DG44	Chinese hamster ovary cell	Large-scale suspension	Host for DHFR coamplification	L. Chasin[b]
CHO DXB11	Chinese hamster ovary cell	Large-scale suspension	Preferred host for DHFR coamplification	L. Chasin[b]
Monkey				
COS-7	Transformed African green monkey kidney cell	Small-scale attachment	Transient expression host	ATCC #CCL-1651
Vero	Normal African green monkey kidney cell	Large-scale attachment	Production of viruses	ATCC #CCL-81

[a]Abbreviation: ATCC, American Type Culture Collection (see *SUPPLIERS APPENDIX*).

[b]E-mail: *lac2@columbia.edu*

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nied by death and rapid lysis of the cell. This presents the purification scientist with the challenge of fishing out the protein of interest, which may be present at ~5 µg/ml, from a soup of lysed cellular protein, nucleic acids, and viral particles. The yield of product during purification may be low as a result of the low titer and starting purity; however, when only small quantities of protein are required, transient expression in COS cells or the vaccinia system is a quick and suitable system to employ.

For production of larger quantities of protein, stable expression must be used because of the difficulty in scaling up transient expression into a bioreactor system. Stable expression in Chinese hamster ovary (CHO) cells is described in *UNIT 5.10*.

Some cell lines have successfully been used as hosts in production of viruses and useful proteins. These include the human embryonic lung cell line MRC-5 and the normal embryonic cell line WI-38. These cells are attachment growth–dependent and have a finite lifespan of ~50 generations, after which time the cells enter a senescent phase and begin to die.

Cell lines that have undergone transformation by virus or that have experienced alteration of chromosomes can exhibit a capacity for infinite growth as well as an ability to grow in suspension. Examples of such cell lines are HeLa cells from a human cervical cancer and Namalwa cells from a human lymphoma. There has been some reluctance to use these cell lines in production of clinical agents because of the possibility of transfer of tumorigenic agents to the product. For production of larger amounts of recombinant protein, stable expression is required and has been primarily performed in CHO cells, baby hamster kidney (BHK-21) cells, or myeloma cells (e.g., NS/O). These cell lines are capable of indefinite growth on a large scale and are suitable hosts for stable integration of heterologous DNA.

Stable expression results from integration into the host-cell genome of the gene for the expression of the heterologous protein. The integrated gene is transcribed efficiently and the protein is expressed persistently over many generations by the host cell. A stable producer-cell line typically provides ~1 to 10 mg of secreted protein per 10^9 cells per day (specific productivity). For monoclonal antibody production, productivity levels of 35 to 100 mg per 10^9 viable cells per day in myeloma cells (Bebbington et al., 1992; Shitara et al., 1994) and 15 to 110 mg per 10^9 viable cells per day in CHO cells (Page and Sydenham, 1991) are obtainable. Such levels may allow secreted-antibody titers of 1 to 1.5 mg/liter to be achieved in optimized large-scale systems; hence these systems have been popular in biotechnology. Where protein is accumulated intracellularly, it may reach little more than 0.1% to 0.5% of total cell protein. Thus, the focus has tended to be on secretion of protein where significant amounts of product are obtainable.

The obvious drawback associated with stable expression in mammalian cells is the time required to obtain stable productive cell banks (months) versus that required to obtain large amounts of non-post-translationally modified proteins in *E. coli* or large quantities of high-mannose-containing glycoprotein using yeast and insect cells (2 to 3 weeks). The advantages of obtaining the more complex proteins from mammalian cells include the ability to understand how these molecules interact in biological models both in vitro and in vivo. Using mammalian systems, much knowledge has been gained in the understanding of post-translational modification, protein quality control, and protein translocation. This knowledge ultimately facilitates improved expression in mammalian hosts.

Stable expression requires the transfer of the foreign DNA, along with the relevant DNA-based signals for transcription by RNA polymerase II, into the chromosomal DNA of the host cell (e.g., CHO). A suitable plasmid vector is required to carry the functions through to the host DNA. Vectors are commercially available from a variety of vendors—e.g., Invitrogen, Clontech, and Promega (see *SUPPLIERS APPENDIX*). A large variety of vectors are described in Pouwels et al. (1988). Suitable replicons contain 5 to 10 kb of DNA and usually are designed to contain phenotypic markers for selection in *E. coli* (e.g., Ap^r) and CHO cells ($DHFR^+$). Other features of appropriate plasmids include a promoter-distal cloning site along with other cloning sites; a replication origin for *E. coli*; a polyadenylation sequence from SV40 or bovine growth hormone; a eukaryotic origin of replication (e.g., SV40 or oriP); the gene for expression of the protein in a site proximal to the promoter; and a promoter and associated elements. Commonly used constitutive promoters for CHO cell applications are the SV40 early promoter (Moreau et al., 1981; Neuhaus et al., 1984), the adenovirus late promoter (McKnight and Tjian, 1986), and the cytomegalovirus (CMV) early promoter (Boshart et al., 1985). Transcriptional enhancers such as the human CMV enhancer (Boshart et al.,

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Gene Expression Using the Vaccinia Virus/ T7 RNA Polymerase Hybrid System

This unit describes a transient cytoplasmic expression system that relies on the synthesis of the bacteriophage T7 RNA polymerase in the cytoplasm of mammalian cells. To begin, a gene of interest is inserted into a plasmid such that it comes under the control of the T7 RNA polymerase promoter (p_{T7}). Using liposome-mediated transfection, this recombinant plasmid is introduced into the cytoplasm of cells infected with vTF7-3, a recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (see Basic Protocol 1). During incubation, the gene of interest is transcribed with high efficiency by T7 RNA polymerase. This transfection protocol is adequate for analytical purposes and is simple because no new recombinant viruses need to be made.

For large-scale work, the p_{T7}-regulated gene can be inserted into a second recombinant vaccinia virus by homologous recombination (*UNIT 5.13*) and used in combination with vTF7-3 to coinfect cells grown in suspension (see Basic Protocol 2) or used to infect OST7-1 cells (a stable cell line that constitutively expresses the T7 RNA polymerase; see Basic Protocol 3). Expressed protein is then analyzed by pulse-labeling (see Support Protocol) or by the methods detailed in *UNIT 5.14*, and purified as described in Chapter 6.

There have been several innovations to this vaccinia virus/T7 RNA polymerase hybrid expression system (see Background Information). One new development is the VOTE inducible expression system, which eliminates the need to use two recombinant viruses or a special cell line (see Basic Protocol 4).

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

NOTE: All reagents and equipment coming into contact with live cells must be sterile, and aseptic technique should be used accordingly.

LIPOSOME-MEDIATED TRANSFECTION FOLLOWING RECOMBINANT VACCINIA VIRUS (vTF7-3) INFECTION

BASIC PROTOCOL 1

This protocol begins with a plasmid vector containing the gene of interest at a site between p_{T7} and the T7 terminator (Figs. 5.15.1 & 5.15.2; also see Critical Parameters). This recombinant plasmid is then introduced via liposome-mediated transfection (*UNIT 5.10*) into cells already infected with vaccinia virus vTF7-3 (a recombinant virus expressing the T7 RNA polymerase gene). After harvesting, the cells are analyzed for expression of the gene product as described in *UNIT 5.14* or by pulse-labeling (see Support Protocol).

Materials

Recombinant plasmid: gene of interest subcloned into pTF7-5 or pTM1 vectors (available from B. Moss, e-mail *bmoss@nih.gov*; Figs. 5.15.1 and 5.15.2) or other plasmid containing the T7 promoter (e.g., pBluescript, Stratagene)
Confluent CV-1 cell monolayer (ATCC #CCL70; *UNIT 5.12*)
Complete DMEM-10 (*APPENDIX 3C*)
vTF7-3 vaccinia virus stock (ATCC #VR-2153)
Opti-MEM I reduced serum medium (Life Technologies)
Liposome suspension (Lipofectin or TransfectAce; Life Technologies)
6-well tissue culture dishes with 35-mm-diameter wells
Cup sonicator (385-W)

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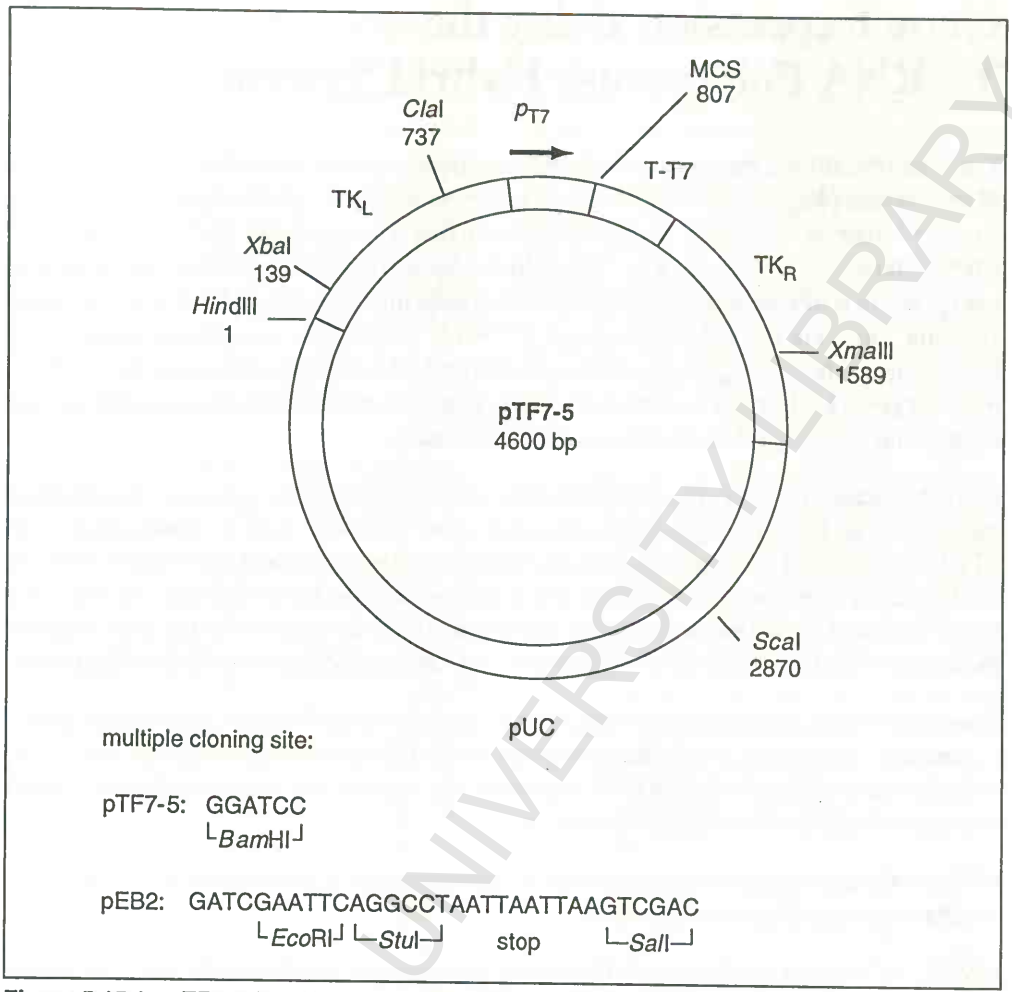

Figure 5.15.1 pTF7-5 (Fuerst et al., 1986) contains p_{T7} and a terminator, between which is a unique *Bam*H1 site for insertion of a gene. The derivative, pEB2 (Berger et al., 1988) contains unique *Eco*RI and *Stu*I sites followed by translation stop codons in all three reading frames. The expression cassette is flanked by segments of the vaccinia virus TK gene; thus, TK^- selection can be used for isolation of recombinant plaques (*UNIT 5.13*).

12 × 75–mm polystyrene tubes (Falcon)

Additional reagents and equipment for purification of plasmid (*APPENDIX 4C*)

1. Purify recombinant plasmid for transfection by CsCl/ethidium bromide centrifugation or PEG precipitation (*APPENDIX 4C*).

   Transfection will require 5 μg DNA/well of cells.

   When using the pTM1 plasmid (Fig. 5.15.2), make sure that the initiator ATG of the gene of interest is the ATG of the pTM1 NcoI site (see Critical Parameters). Determine that the correct construction has been obtained by restriction mapping and DNA sequencing.

2. The day before infection, seed wells of a 6-well tissue culture dish with 5×10^5 CV-1 cells/well in complete DMEM-10. Incubate until cells are near confluency (usually overnight).

   Cells should be counted with a hemacytometer (APPENDIX 3C).

3. Vortex stock of vTF7-3 to disperse clumps. Sonicate the lysate using a cup sonicator as follows.

   a. Fill the cup with ice water (~50% ice).

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Stern-Volmer equations to give the quenching constant (K_{SV}) and the fraction of the initial fluorescence that is accessible to the quencher (see Basic Protocol 1).

The quenching of fluorescence of phosphoglycerate kinase (PGK) is shown plotted according to the Stern-Volmer equation (see Fig. 7.7.6A). The quenching levels off above 0.9 M succinimide and 0.6 M iodide, respectively, indicating that part of the fluorescence is unquenched by these quenchers. The different degrees of quenching by iodide and succinimide is due to the nature of the neighboring groups affecting access to the tryptophan. The plot of some of the data according to the modified Stern-Volmer equation (Fig. 7.7.6B) shows the fluorescence quenched by succinimide to be 42% of the total. The different degrees of quenching at 316 and 350 nm are due to the two tryptophans having substantially different values of λ_{max}, and hence different degrees of accessibility to solvent. Thus, taking into account the crystal structure of PGK, one of the two tryptophans, W333, is seen to be buried and inaccessible to quencher while the other, W308, is quenched with a quenching constant of $K_{SV} = 0.39$ liters/mol.

Another quencher, acrylamide, can quench the fluorescence not only of exposed but also of buried tryptophan residues based on its ability to diffuse through the protein structure. The efficiency of quenching is, therefore, a reflection of the tightness of packing and the dynamics of the protein. Figure 7.7.11 shows the results of acrylamide quenching of the

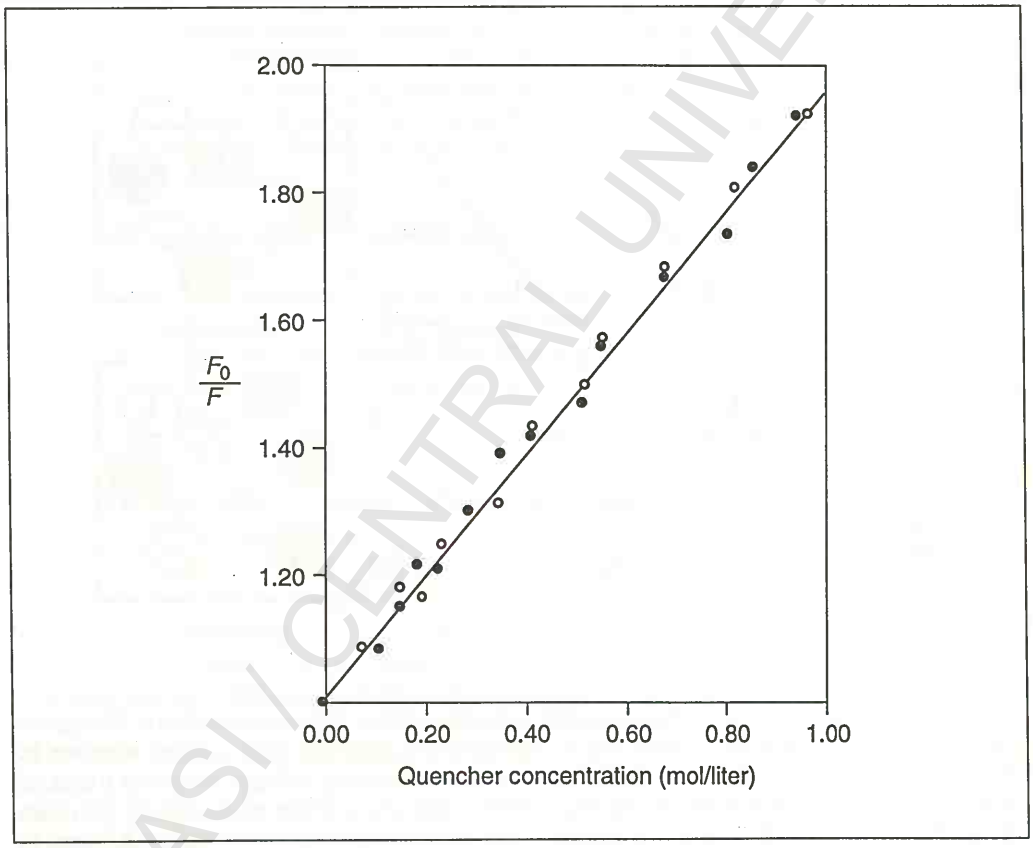

Figure 7.7.11 The quenching of buried tryptophan fluorescence in 3-phosphoglycerate kinase (PGK). In the presence of 1.12 M succinimide (shown to quench the exposed tryptophan fluorescence; see Fig. 7.7.6), fluorescence is further quenched by acrylamide, which is able to gain access to the buried tryptophan. Fluorescence was measured at 316 nm (filled circles) and at 350 nm (open circles) and plotted according to the Stern-Volmer equation (see Basic Protocol 2, step 4). Fluorescence intensities were corrected for dilution and for quencher absorbance using $\varepsilon_{295} = 0.236$ for acrylamide. The linear plot indicates the quenching of a single residue. Measurements made using a Perkin-Elmer MPF3-L. Reprinted from Varley (1991) with permission of Elsevier Science.

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buried W333 of PGK (in this experiment the more exposed W308 is quenched by succinimide). The linearity of the plot, combined with the coincidence of the quenching measured at 316 and 350 nm (in contrast to Fig. 7.7.6), shows that a single tryptophan only is being quenched by the acrylamide. A quenching constant $K_{SV} = 0.94$ liters/mol is obtained from the slope. In general, bimolecular rate constants for quenching, which reflect more directly the dynamics of the protein, can also be calculated if fluorescence lifetime measurements are available (see discussion of Stern-Volmer equation in Basic Protocol 2; see also Eftink and Ghiron, 1981).

Fluorescence Depolarization

If a polarizer is placed in the excitation beam, the radiation passing through the sample solution will be plane-polarized. This beam will now be absorbed only by those chromophores whose transition dipoles are in the same plane (see Fig. 7.7.12). If the chromophore does not rotate within 10^{-8} sec (see Fig. 7.7.7), the fluorescence will be emitted with the same degree of polarization and will pass through a second polarizer (or analyzer) oriented parallel to the first polarizer. If, on the other hand, the excited chromophores rotate before fluorescence takes place, emission will take place from a smaller number of molecules reoriented with transition dipoles parallel and perpendicular to the exciting radiation

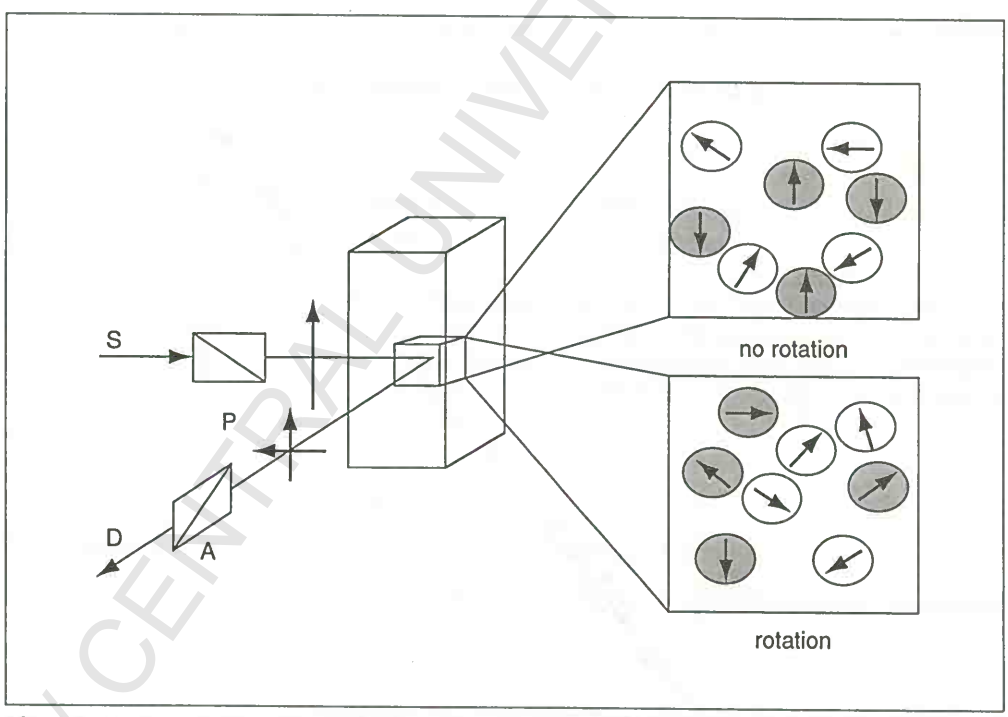

Figure 7.7.12 Depolarization of fluorescence indicates rotation of the chromophore. Monochromatic radiation from the source (S) has all but the vertically polarized electric vector removed by the polarizer (P). This is absorbed only by those molecules (see Fig. 7.7.5) in which the transition dipole of the chromophore is aligned vertically. In the case where these molecules do not rotate appreciably before they fluoresce ("no rotation"), the same molecules will fluoresce (indicated by shading) and their emitted radiation will be polarized parallel to the incident radiation. The intensity of radiation falling on the detector (D) will be zero when the analyzer (A) is oriented perpendicular to the polarizer. In the case where the molecules rotate significantly before fluorescence takes place, some of the excited chromophores will emit radiation with a horizontal polarization ("rotation") and some with a vertical polarization. Finite intensities will be measured with both parallel and perpendicular orientations of the analyzer. The fluorescence from the remainder of the excited molecules will not be detected. The heavy arrows on the left of the diagram illustrate the case where there is rotation.

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CONVENTIONAL CHROMATOGRAPHIC SEPARATIONS

8

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Gel-Filtration Chromatography

Gel filtration (GF) separates proteins solely on the basis of differences in molecular size. Separation is achieved using a porous matrix to which the molecules, for steric reasons, have different degrees of access (smaller molecules have greater access and larger molecules less). The matrix is packed into a chromatographic column, the sample mixture applied, and the separation accomplished by passing an aqueous buffer (the mobile phase) through the column. Protein molecules that are confined in the volume outside the matrix beads will be swept through the column by the mobile phase and the sample is thus eluted in decreasing order of size. The protein zones eluted are detected by an in-line UV monitor and fractions are collected for subsequent specific analysis or further preparation steps.

GF is often used to condition samples prior to further purification by adsorptive techniques. It is also frequently used in the final polishing stage (*UNIT 8.1*) to remove a few remaining contaminants with surface properties similar to the target protein (e.g., for polishing monomers from oligomers).

Three basic protocols are provided in this unit, corresponding to the major applications of gel filtration. GF is used for desalting or group separation (see Basic Protocol 1), in which the target protein and contaminating solutes differ substantially in molecular size. It is also used for protein fractionation (see Basic Protocol 2), where proteins with only a small size difference must be separated. A third application is in characterizing the molecular dimensions of proteins (see Basic Protocol 3). A Support Protocol is also provided for calibrating GF columns to be used in estimating molecular size.

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STRATEGIC PLANNING

Selecting Matrix and Column for Desalting

The exclusion limit—i.e., the smallest-sized protein molecule that will be excluded from the pores of the matrix—is the most important factor in selecting a matrix for desalting. If the protein is able to permeate the matrix, flow-dependent zone broadening (*UNIT 8.1*) will occur, reducing the resolution. On the other hand, gels of very small pore size generally contain more matrix, and thus have smaller separating volumes. A gel of sufficiently small exclusion limit, but not smaller, should therefore be selected. Another factor to take into consideration is the particle size of the matrix. This should not be too small because the pressure drop over the column is inversely proportional to the square of the particle size; this factor is especially important for gravity flow columns.

The selection of column dimensions (i.e., the bed volume of the column) is influenced by the amount of sample that is to be processed. Columns for desalting are typically short and wide to accommodate large sample volumes and keep the processing time short. Because the zone broadening of the column will have a limited effect on the peak width in desalting as compared to other applications of gel filtration—e.g., protein fractionation—short columns run at high flow rates are used for large-scale desalting. The typical length of columns for laboratory use is ~10 cm.

Columns for desalting vary greatly in size and sophistication, ranging from Pasteur pipets plugged with glass wool to prepacked high-performance GF columns. The scale of

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Table 8.3.1 Gel Filtration Matrices Suitable for Desalting[a]

Gel type	Exclusion limit		Supplier[d]
	M_r[b]	Radius (Å)[c]	
Sephadex G-10	700	7	PB
Bio-Gel P-2	1,800	10	BR
Sephadex G-25	5,000	14	PB
Bio-Gel P6DG	6,000	15	BR
Sephadex G-50	30,000	25	PB
Bio-Gel P-30	40,000	28	BR

[a]Data as reported by manufacturers.

[b]Molecular mass of smallest protein excluded from matrix.

[c]Apparent radius of smallest spherical protein excluded from matrix, calculated as $R = 0.808\ M_r^{1/3}$, where R is the radius and M_r the molecular mass (Hagel, 1989).

[d]Abbreviations: BR, Bio-Rad; PB, Amersham Pharmacia Biotech. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

operation for which they are used also varies widely, with applications ranging from micropreparative cleanup of a 50-µl reduction/alkylation mixture using a 0.9-ml column to industrial de-ethanolization of 12 liters of human serum albumin using a 75-liter column. Incidentally, columns for both these procedures may be packed with Sephadex G-25—of course, of different particle size.

Some gel-filtration media suitable for laboratory-scale desalting are listed in Table 8.3.1, and some empty columns that can be used with these media are listed in Table 8.3.2. A partial compilation of disposable prepacked columns for desalting is found in Table 8.3.3. This table also contains information about spin columns for use with a centrifuge. Spin columns require special techniques, and they are presently not widely used for desalting proteins; therefore no protocols for their use have been included in this unit. More information about spin columns can be obtained from the suppliers.

Selecting Matrix and Column for Protein Fractionation

Suitability of a gel for protein fractionation may be judged from "typical runs" made with synthetic protein mixtures, the results of which are published by suppliers. The solute of interest should ideally be eluted in the first part of the chromatogram, but not too close to the void volume. A matrix with a distribution coefficient (see Basic Protocol 3) of ~0.2 to 0.4 has been reported to yield maximum resolution (Hagel, 1989). This corresponds to an elution volume of ~1.5 to 2 times the void volume. A long bed and a matrix with a large pore volume will increase the peak-to-peak distance between the proteins and thus increase the resolution of the system (also see Critical Parameters and *UNIT 8.1*). Another way to increase resolution is to employ a GF matrix of small particle size, which will reduce the zone width of the protein bands. The flow rate will also affect resolution. As in the case of desalting, the maximum sample volume that can be processed depends upon the bed volume and the pore volume of the packed bed. Thus, there are many parameters to consider when optimizing protein fractionation by GF. In the worst case, a suitable matrix may have to be found by trial and error.

Commercial prepacked columns for protein fractionation often sacrifice column length and pore volume in favor of small particle size. This results in columns that are optimized for fast separation at the expense of some reduction in the maximum sample volume that can be loaded. Therefore, traditional columns packed in the laboratory are often still a

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good alternative for purification of large volumes of sample. If time is critical in the separation (e.g., in proteinase removal), fast column operation is of course advantageous. Matrices of large particle size will permit protein fractionation at low operating pressure. Some matrices suitable for protein fractionation are listed in Table 8.3.4, and some empty columns suitable for use with these media are listed in Table 8.3.5. Table 8.3.6 lists some prepacked columns for protein fractionation. Columns and media are selected on the basis of the difficulty of the separation (i.e., how small the size difference is between the proteins to be separated) and whether scaleup will be required (i.e., the availability of a variety of

Table 8.3.2 Empty Columns Suitable for Desalting[a]

Column	Minimum bed volume (ml)	Maximum bed volume (ml)	Maximum operating pressure (MPa)	Supplier[b]
PD-10[c]	1	15	—	PB
Econo-Pac[c]	1	20	—	BR
C series[d]	0	520	0.1	PB
Econo-Column[d]	1	1374	0.1	BR
XK series[d]	2	1884	0.5	PB

[a]Data as reported by the manufacturers.
[b]Abbreviations: BR, Bio-Rad; PB, Amersham Pharmacia Biotech. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.
[c]Plastic, disposable.
[d]Borosilicate glass.

Table 8.3.3 Prepacked Columns Suitable for Desalting[a]

Prepacked column	Gel type	Exclusion limit		Maximum sample volume (ml)	Supplier[d]
		M_r[b]	Radius (Å)[c]		
Columns for use with a pump					
Hi Trap Desalting	Sephadex G-25 SF	5,000	14	1.5	PB
Fast Desalting HR 10/10	Sephadex G-25 SF	5,000	14	3.0	PB
Econo-Pac P6	Bio-Gel P-6	6,000	15	3.0	BR
Gravity-flow columns					
NAP-5	Sephadex G-25 M	5,000	14	0.5	PB
NAP-10	Sephadex G-25 M	5,000	14	1.0	PB
PD-10	Sephadex G-25 M	5,000	14	2.5	PB
Econo-Pac 10DG	Bio-Gel P-6DG	6,000	15	3.0	BR
Spin columns					
MicroSpin S-200	Sephacryl S-200 HR	250,000	51	0.01-0.05	PB
Bio-Spin 6	Bio-Gel P-6	6,000	15	0.05-0.1	BR
Nick Spin Column	Sephadex G-25 SF	5,000	14	0.075-0.15	PB
Bio-Spin 30	Bio-Gel P-30	40,000	28	0.05-0.1	BR

[a]Data reported by the manufacturer.
[b]Molecular mass of smallest protein excluded from matrix.
[c]Apparent radius of smallest spherical protein excluded from matrix, calculated as $R = 0.808\ M_r^{1/3}$, where R is the radius and M_r is the molecular mass (Hagel, 1989).
[d]Abbreviations: BR, Bio-Rad; PB, Amersham Pharmacia Biotech. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

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columns with different diameters that can be packed with bulk media facilitates linear scaleup).

A matrix of small particle size will permit preparation of columns of high efficiency (i.e., low zone broadening) at the expense of high pressure drops and greater cost. Also, smaller particles generally have smaller pore volumes. The net effect of the relationships between these parameters is that the prepacked columns listed in Table 8.3.6 have resolving power of the same order of magnitude, although protein fractionation may be carried out faster on the columns that have particles of small size.

A final factor to consider in choosing a matrix for protein fractionation is the likelihood of matrix-solute interactions (e.g., as in the case of lectins on gels containing glucose-type structures).

Selecting Matrix and Column for Molecular Size Determination

A matrix with high resolving power is recommended for determination of molecular size as well as for protein fractionation (see Table 8.3.4, Table 8.3.5, and Table 8.3.6 for

Table 8.3.4 Gel-Filtration Matrices for Protein Fractionation or Size Determination[a]

Gel type	Fractionation range (M_r)	Particle size (μm)	Supplier[b]
Toyopearl HW 40S	100-10,000	25-40	TH
Superdex 30 prep grade	200-10,000	34	PB
Toyopearl HW 50S	500-80,000	25-40	TH
Sephacryl S-100 HR	1,000-100,000	47	PB
Toyopearl HW 55S	1,000-700,000	25-40	TH
Superdex 75 prep grade	3000-70,000	34	PB
Sephacryl S-200 HR	5,000-250,000	47	PB
Superose 6 prep grade	5,000-5,000,000	34	PB
Superdex 200 prep grade	10,000-600,000	34	PB
Sephacryl S-300 HR	10,000-1,500,000	47	PB
Sephacryl S-400 HR	20,000-8,000,000	47	PB
Sephacryl S-500 HR	20,000-30,000,000	47	PB
Toyopearl HW 65S	50,000-5,000,000	25-40	TH
Toyopearl HW 75S	500,000-50,000,000	25-40	TH

[a]Data as reported by manufacturers.

[b]Abbreviations: PB, Amersham Pharmacia Biotech; TH, Toso Haas. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

Table 8.3.5 Empty Columns Suitable for Protein Fractionation or Size Determination[a]

Columns	Dimensions[b] (length × inner diameter, cm)	Maximum operating pressure (MPa)	Supplier[c]
HR series	20 × 0.5–50 × 1.6	3–5	PB
G series	25 × 1.0–100 × 4.4	3-7	AM
XK series	20 × 1.6–100 × 5	0.5	PB

[a]Data as provided by manufacturers. All columns are made of borosilicate glass.

[b]Column dimensions given here are those suitable for protein fractionation and size determination. Other column dimensions may also be available.

[c]Abbreviations: AM, Amicon; PB, Amersham Pharmacia Biotech. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

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Table 8.3.6 Prepacked Columns for Protein Fractionation or Size Determination[a]

Column type[b]	Fractionation range (M_r)[c]	Particle size (μm)	Dimensions available (length × inner diameter, cm)	Supplier[d]
Superdex Peptide	100-7,000	13	30 × 1	PB
HiLoad Superdex 30 prep grade	200-10,000	34	60 × 1.6 60 × 2.6	PB
TSK SW 2000	500-60,000	10	30 × 0.75 60 × 0.75	TH
HiLoad Sephacryl S-100 HR	1,000-100,000	47	60 × 1.6 60 × 2.6	PB
TSK SW 3000	1,000-300,000	10	30 × 0.75 60 × 0.75	TH
Protein-Pak 60	2,000-30,000	10	30 × 0.78	WA
Superdex 75 HR 10/30	3,000-70,000	13	30 × 1	PB
HiLoad Superdex75 prep grade	3,000-70,000	34	60 × 1.6 60 × 2.6	PB
Bio-Sil SEC 125	5,000-100,000	10	60 × 0.75 60 × 2.15	BR
$G2000SW_{XL}$	5,000-150,000	5	30 × 0.75	TH
HiLoad Sephacryl S-200 HR	5,000-250,000	47	60 × 1.6 60 × 2.6	PB
TSK SW 4000	5,000-1,000,000	13	30 × 0.75 60 × 0.75	TH
Superose 6 HR 10/30	5,000-40,000,000	13	30 × 1	PB
Protein-Pak 125	10,000-80,000	10	30 × 0.78	WA
Glass GF-250	10,000-250,000	4	30 × 1	DU
Bio-Sil SEC 250	10,000-300,000	10	60 × 0.75 60 × 2.15	BR
$G3000SW_{XL}$	10,000-500,000	5	30 × 0.75	TH
Superdex 200 HR 10/30	10,000-600,000	13	30 × 1	PB
HiLoad Superdex 200 prep grade	10,000-600,000	34	60 × 1.6 60 × 2.6	PB
HiLoad Sephacryl S-300 HR	10,000-1,500,000	47	60 × 1.6	PB
$G4000SW_{XL}$	20,000-10,000,000	7	30 × 0.75	TH
Glass GF-450	25,000-900,000	6	30 × 1	DU

[a]Data as reported by the manufacturers.

[b]In general, matrices of natural polymers (e.g., Superdex, Sephacryl, or Superose) have larger pore volumes than silica-based materials (e.g., TSK SW, Protein-Pak, Bio-Sil SEC, or GF-250/450).

[c]Selectivity of materials decreases with increased width of the fractionation range and also with decreased pore volume.

[d]Abbreviations: BR, Bio-Rad; DU, DuPont; PB, Amersham Pharmacia Biotech; TH, Toso Haas; WA, Waters. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

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matrices and columns suitable for both these applications). However, certain other criteria that are important for selecting a matrix and column for size determination differ from those for protein fractionation. First, when analyzing samples containing several proteins of varying molecular size (e.g., protein digests) a matrix of sufficient separation range is needed. As selectivity inherently limits separation range, optimal separation range may not be achieved using a matrix of high selectivity. Second, because peak broadening is not critical as long as the peaks are resolved, short (e.g., 30-cm) columns operated at high flow rates may be used. However, in situations where the effluent is continuously monitored with a mass-sensitive detector (see Basic Protocol 3), complete resolution between peaks is essential. In this case the criteria used for protein fractionation may be applied.

BASIC PROTOCOL 1

DESALTING (GROUP SEPARATION)

Desalting, or group separation, is used to separate the target protein from low-molecular-mass contaminants. The purpose might be to remove salt prior to lyophilization, or to remove low-molecular-mass reagents—e.g., a reductive cleavage mixture from a preparation step (*UNIT 11.1*) or polybuffer from chromatofocusing (*UNIT 8.5*). Desalting is also used to change from one buffer to another—e.g., to decrease ionic strength prior to ion-exchange chromatography (*UNIT 8.2*). The pore size of the matrix is selected to exclude the protein of interest while allowing maximum permeation of the contaminants. This is beneficial in three ways. First, because the protein does not penetrate into the pores, little dilution of the sample will take place except where the sample volume is very small. Second, because the protein is unable to diffuse into the pores, no flow-sensitive zone broadening (*UNIT 8.1*) that results from slow mass-transfer will take place. Hence, the flow rate may be kept high during the desalting. Third, because the protein and the low-molecular-mass solutes are maximally separated, very large sample volumes may be applied before contamination of the protein band becomes evident. Thus, very large sample volumes may be applied and desalted in a short time without dilution of the sample.

The procedure described here uses a closed column system and a pump; annotations explain variations needed in some of the steps if an open-bed column and gravity flow are used. The GF matrix is swelled, the column is packed, and the void volume and total volume of the column are determined in preliminary runs using high- and low-molecular-weight solutes. This information is used to determine the maximum sample volume to apply for the unknown protein to be desalted. Guidelines for estimating approximate sample volumes to use for desalting are also given. Alternative steps are provided for using this procedure either to determine elution volume or to desalt a protein for preparative purposes (or further analysis). Elution volumes must be determined to ascertain the exact void volume and total volume of a column in preparation for desalting. It is also necessary to measure the elution volume for certain protein analyses—e.g., for determining molecular size (also see Basic Protocol 3).

Materials

GF matrix (Table 8.3.1) or prepacked GF column (Table 8.3.3) with appropriate exclusion limit for protein of interest (also see Strategic Planning and see Critical Parameters)
GF desalting buffer (see recipe)
Colored marker: 0.2 mg/ml Blue Dextran 2000 or 0.2 mg/ml vitamin B_{12}
Void marker (see recipe)
Total liquid volume marker (see recipe)
Protein sample to be desalted

90°C water bath (optional)
Buchner or sintered-glass funnel

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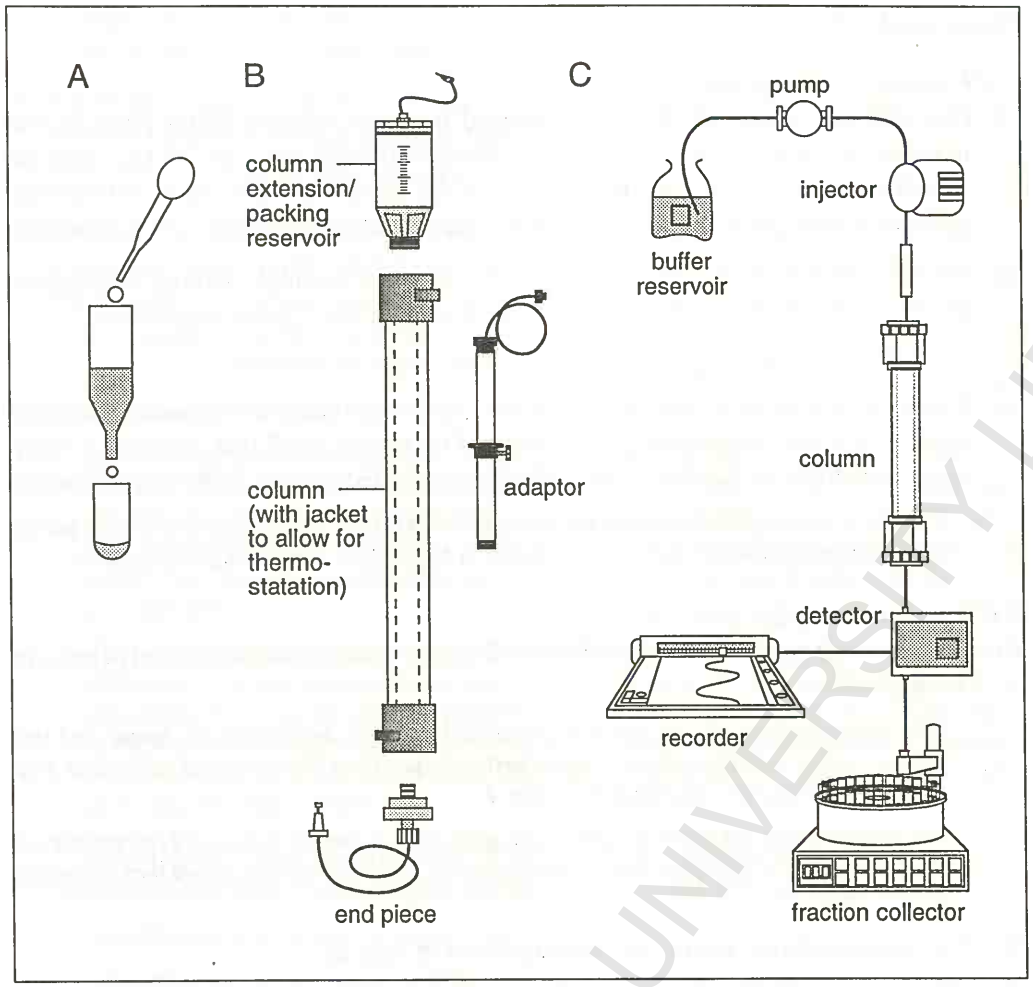

Figure 8.3.1 Equipment used for gel filtration. **(A)** Simple setup for desalting using an open-bed column made with a Pasteur pipet. **(B)** Column and attachments. **(C)** Complete automated chromatography system. Courtesy of Amersham Pharmacia Biotech.

GF chromatography column (see Critical Parameters and Table 8.3.2) with column extension (optional), adaptors, and buffer reservoir (Fig. 8.3.1)
Carpenter's level
Peristaltic pump (Fig. 8.3.1)
0.22-μm filter: any 0.22-μm filter for buffer; protein-compatible for sample
Detector (optional; Fig. 8.3.1)
Chart recorder (optional; Fig. 8.3.1)
Sample applicator: sample application loop (e.g., Superloop, Amersham Pharmacia Biotech) or syringe
Fraction collector (optional; Fig. 8.3.1)

NOTE: Small-scale desalting of multiple samples may be carried out using spin columns and a centrifuge. At present, spin columns are primarily used for preparing microliter-sized samples of oligonucleotides; however, they are equally useful for small-volume protein samples. As the procedure is different from that described here, manufacturer's instructions should be consulted (see Table 8.3.3 for suppliers).

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Prepare gel

If GF matrix is supplied dry:

1a. Calculate the amount of dry matrix needed from the swelling factor given by the manufacturer and the approximate bed volume of the column to be packed. Add the calculated amount of dry gel to a volume of GF desalting buffer equal to twice the calculated final gel volume (i.e., twice the approximate bed volume of the column).

2a. Carefully stir suspension with a glass rod. Let gel swell overnight at room temperature or for 3 hr in a 90°C water bath, stirring as needed to keep gel in suspension.

Do not use a magnetic stirrer, as this may break fragile gel particles.

3a. Allow gel bed to settle, then remove fines and broken beads by decanting the hazy solution. Repeat decantation (4 or 5 times if necessary) until bed settles as a sharp zone, then dilute so that final slurry is 50% (v/v) settled gel and 50% (v/v) GF buffer.

Careful removal of fine particles will prevent the nets that support the column end pieces from becoming blocked, and will also permit a higher flow rate through the column.

If GF matrix is supplied preswollen:

1b. Wash gel with an excess of GF buffer on a Buchner or sintered-glass funnel to remove storage buffer.

With some products, the gel slurry is obtained by simply shaking the gel bottle, and this can be used to pack the column without further preparation. If this type of gel is used, skip steps 1b to 3b and proceed directly to step 4.

Gel particles may adhere to the glass wall of the funnel; therefore, to avoid contamination from other types of gels (e.g., ion-exchange media), it is important to ensure that glassware used is clean.

2b. If gel contains fines, remove by decantation as in step 3a.

3b. Dilute so that final slurry is 50% (v/v) settled gel and 50% (v/v) GF buffer.

Pack column

4. Examine column to ensure that it is clean and that the support nets are undamaged. If necessary, supply column with new support nets.

5. Mount column on a stable laboratory stand, using a carpenter's level to ensure that it is vertical. Equip column with an extension that together with the column will hold the complete volume of the gel slurry (i.e., twice the volume of the settled gel).

If <50% of the column volume is to be packed, there is no need for a column extension.

6. Remove air from outlet tubing and end piece of column by injecting GF buffer into outlet tubing (using syringe or peristaltic pump) until support net is covered with 0.5 cm of buffer. Close outlet of column.

7. Inject GF buffer into inlet tubing of adaptor until net is wetted, while holding outlet upwards to ensure that air easily escapes from net. Place adaptor in a beaker containing GF buffer until it is time to use it.

8. Swirl gel slurry from step 3a or b and pour entire slurry into column along a glass rod tilted toward the inner wall of the column or column extension. Fill the remaining space with GF buffer by carefully pouring buffer along glass rod so as to cause minimum disturbance of the gel layer. Put lid on column extension (or put top adaptor on column).

Small columns (e.g., 1-cm i.d., 5-cm length) to be used for preliminary experiments where optimum resolution is not critical may be quickly prepared by adding the gel slurry to the

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chromatographic tube, letting the gel settle by gravity, and washing the bed with one column volume of GF buffer before applying the sample.

9. Fill buffer reservoir with GF buffer and place at a level higher than the pump. Connect reservoir to pump using a large-i.d. tube.

   This step is to ensure a free flow of buffer to the pump head.

10. Purge pump with GF buffer and attach outlet from pump to inlet of column or column extension. Open outlet of column and start pump at flow rate recommended by manufacturer for the gel/column combination used. Continue flow until height of gel bed becomes constant (typically 1 to 4 hr).

    If no manufacturer's recommendation is available, use a flow rate 50% higher than that to be used in the separation.

    If column is to be packed by gravity flow—i.e., by attaching the buffer reservoir directly to the inlet of the column—the actual flow rate must be monitored (e.g., by continuously weighing the effluent, or in the simplest case, by using a graduated cylinder) or controlled by a pump placed after the column. In the latter case it is necessary to check that the free gravity flow exceeds the pump flow—i.e., that the pump is serving to reduce the flow to a constant value.

    The flow rate caused by gravity depends upon the hydrostatic pressure, determined by the difference in height between the air inlet in the buffer reservoir and the outlet tubing from the column. However, because the pressure drop over the column increases with the height of the packed bed, the free gravity flow rate will decrease during packing if the hydrostatic pressure is kept constant. Gravity flow rate may be increased by lowering the outlet tube or raising the buffer reservoir.

11. Turn pump off, close column outlet, remove column extension, adjust bed height if necessary, and adjust inlet adaptor so that it is flush with the bed surface.

    Avoid trapping air under the support net.

    Adjustment of the bed height is recommended only to remove excess gel. This is done by carefully swirling the upper part of the gel surface with a spatula and sucking the excess slurry out with a Pasteur pipet or tubing attached to a pump. If a crater or uneven surface is produced, the gel should be swirled so that a sharp bed surface is obtained before adjusting the inlet adaptor atop the bed. If the bed is too short the column should be repacked, although in most cases the old gel can be reused and added to new slurry prepared as in steps 1a to 3a or 1b to 3b before repacking.

12. Reattach column to pump, open column outlet, and resume flow conditions used in step 10 for 1 hr to stabilize column bed height. Readjust the adaptor so that it is flush with the new bed surface.

    In some cases it is desirable to lower the inlet adaptor a few millimeters into the gel surface to make sure that there is contact between adaptor and gel.

    Stabilizing the bed at a higher flow rate than that to be used during the separation will prevent the bed from shrinking as a result of "after-packing" during the run. If a gap appears between the adaptor and the bed surface, step 12 must be repeated at an increased flow rate before experiments are carried out. A space between the adaptor and the bed surface will act as a mixing chamber; the effect on separation will depend on the separation problem at hand.

13. Inspect packed bed visually for cracks, trapped air, and particle aggregates by observing the light scattered from a light source held behind the column. Chromatograph a colored marker (2 mg/ml Blue Dextran 2000 or 0.2 mg/ml vitamin B_{12}) as in steps 20 to 23, and ascertain that the zone produced is horizontal and sharp.

    Quality of the packed bed need not be as high for desalting as for protein fractionation (see Basic Protocol 2); therefore evaluation of the packed bed may be performed by visual

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inspection. However, it is important for it to be free of cracks and for a colored sample to produce a sharp horizontal zone.

Air bubbles would be a major problem and should be avoided at all costs (see Troubleshooting).

If column is to be stored, GF buffer containing 0.02% (w/w) sodium azide or 20% (v/v) ethanol as antibacterial agents should be prepared, then 2 column volumes of this mixture should be pumped through the column. The outlet is then closed and the column stored in the buffer/antibacterial agent at room temperature for several months. See recipe for GF buffer for precautions concerning azide.

Prepare and test the GF system

14. Calculate amount of GF buffer necessary for the run and filter this amount plus a 50% excess through a 0.22-μm filter. Adjust pH if necessary and pour filtered buffer into buffer reservoir.

    If the column is going to be used for a prolonged time, it is good practice to continuously deaerate the GF buffer by heating the buffer slightly with heat from a light bulb, or to use a degasser.

15. Assemble GF system according to manufacturer's instructions or Figure 8.3.1, placing detector and chart recorder (if used) in line but leaving column off line. Attach buffer reservoir to pump and purge pump with GF buffer with column off line.

    If the system is run by gravity flow alone, the risk of the column running dry may be eliminated by placing the end of the outlet tubing higher than the inlet adaptor or placing a loop of the tubing from the reservoir with its bend at a lower level than the outlet.

    Detection of salt may be accomplished in line by conductivity or off line by flame photometry or conductimetry. Colorimetric detection of proteins is readily done at 280 or 254 nm; the latter wavelength provides higher sensitivity. Refer to Chapter 3 for specific methods of protein detection.

16. Connect outlet of pump to column via the injection valve, run system with GF buffer at flow-rate settings to be used for separation, collect fractions with fraction collector, and note actual flow rate of the pump (e.g., by weighing collected fractions or measuring them with a graduated cylinder).

    If the concentration of the solute is detected by in-line monitoring, use of a two-channel recorder set at different sensitivities (e.g., 100% and 10% full-scale) is recommended to ensure that the peaks of unknown solutes are not off-scale.

    The actual flow rate of the system must be determined with the column installed, because the flow rate from a peristaltic pump or similar device depends upon the back-pressure of the system.

Determine separating volume of column

17. Determine the void volume of the system (V_o) by running a void marker and obtaining the elution volume according to steps 20 to 23.

    The sample volume for determination of void volume and total liquid volume (steps 17 and 18) is not critical and may typically be 1% to 2% of the bed volume. However, correction of the calculated elution volume is needed if large sample volumes are applied (see annotation to step 23a). See Figure 8.3.2 for graphical depiction of void and total liquid volume.

    The markers for void volume and total liquid volume may be mixed and run together.

    Void volume (V_o) is typically 30% to 33% of the bed volume of a nonrigid (e.g., agarose) gel and 36% to 40% of the bed volume of a rigid (e.g., silica) gel.

18. Determine the total liquid volume of the system (V_t) by running a total liquid volume marker and obtaining the elution volume according to steps 20 to 23.

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Total liquid volume (V_t) is typically 85% to 95% of the geometric bed volume ($\pi r^2 L$, where r is the inner radius of the column) for a nonrigid gel and 70% to 80% of the bed volume for a rigid gel. Because solute-matrix interactions may occur, it is advisable to check that the total liquid volume is smaller than the bed volume and perhaps run several different total liquid volume markers to confirm the result.

19. Calculate the separating volume of the column (V_i) as $V_t - V_o$.

The separating volume (V_i) is the amount of liquid that will pass through the column from the point where large proteins begin to emerge to the point where small solutes are eluted. It is equal to the pore volume of the bed, which is typically 50% to 65% of the bed volume of a nonrigid gel and 30% to 50% of the bed volume of a rigid gel.

Prepare, apply, and chromatograph the sample

20. Dissolve sample to be desalted in GF buffer. Filter through a 0.22-µm protein-compatible filter.

If possible, let sample solution reach room temperature before being applied to column to reduce viscosity effects. This is more important for large sample volumes.

The influence of the sample volume on the resolution (see Critical Parameters) is related to the need for column efficiency (see Basic Protocol 2 and UNIT 8.1). Too large a sample volume may ruin the separation of closely eluting proteins in a separation where high efficiency and narrow peaks are required. This is of primary interest in protein fractionation (see Basic Protocol 2), whereas in desalting, conditions are chosen to yield maximum separation of the protein from contaminants. To this end, column efficiency is less important, and very large sample volumes may be applied. See Critical Parameters for guidelines on optimal sample concentration.

When GF is performed for analytical purposes, the sample concentration should be as small as possible within the sensitivity of the detection method employed. For preparative purposes, on the other hand, the concentration should be as high as possible. The limiting factor in the latter case is the viscosity of the sample divided by the viscosity of the GF buffer (relative viscosity). At relative viscosities >1.5, the hydrodynamic instability of the trailing edge of the zone may cause distortion of the protein zone (resulting from invasion of less viscous eluant into the more viscous zone; called "viscous fingering"), which will ruin the separation. It is generally safe to use protein concentrations up to 50 mg/ml and in some cases up to 70 mg/ml, whereas concentrations of 100 mg/ml often result in viscous

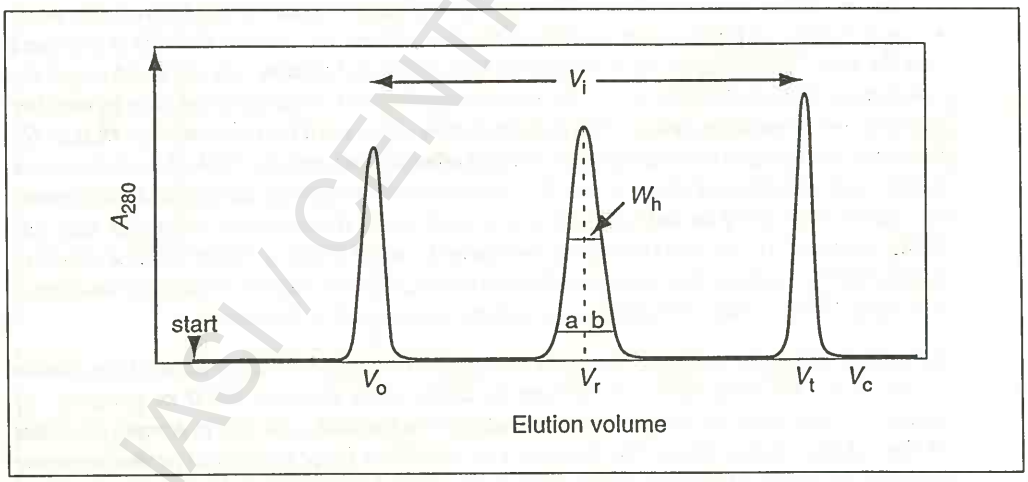

Figure 8.3.2 Chromatogram illustrating the separating volume (V_i) of gel filtration as determined by the void volume (V_o) and the total liquid volume (V_t). The total bed volume (V_c) is equal to the total liquid volume plus the matrix volume. Column efficiency is calculated as $N = 5.54(V_r/W_h)^2$, where N is the efficiency of the column in terms of number of theoretical plates, V_r is the elution volume of the peak, and W_h is the peak width at half peak height. The symmetry of the peak is calculated at 10% peak height as b/a, where b is the width of the tailing part of the peak and a is the width of the leading part of the peak.

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fingering. However, when large sample volumes are applied—e.g., in desalting—the low dilution factor will result in a high concentration of protein in the peak, hence lower concentrations than these may be required to avoid viscosity effects.

21. Open outlet from column, turn on pump, and let two bed volumes of GF buffer pass through the column. Turn on detector and recorder and allow baseline to stabilize.

22. Load sample applicator with the appropriate volume of sample for desalting, not exceeding the separating volume determined in step 19. Switch the sample application valve to the load position and put a start mark on the chart recorder paper (or start a stopwatch if no chart recorder is used).

    Smaller sample volumes are used for protein fractionation than for desalting, and much smaller sample volumes are used for analysis.

    Before injecting, flush injector loop with excess sample to avoid trapping air bubbles, which otherwise may end up in the top of the column.

    If the separating volume of the column has not been determined, the maximum sample volume may be estimated as ~50% of the bed volume for nonrigid media and ~30% of the bed volume for rigid media. For a first attempt at desalting, 50% of these volumes should be applied (i.e., 25% and 15% of the bed volume for nonrigid and rigid media, respectively).

    If an open-bed column is used, gently add GF buffer to the top of the column with a Pasteur pipet or similar device, then open the outlet from the column and continue adding GF buffer until two bed volumes of buffer have passed through. Let the last portion of buffer penetrate the gel without the gel surface becoming dry; this is facilitated by using a top filter on the bed. Gently apply the sample to the clear gel surface or the top filter using a micropipet (or a Pasteur pipet if large volumes of sample are to be desalted).

23a. *To determine elution volume:* Pass GF buffer through the system at the appropriate flow rate and follow detector response with recorder while collecting fractions. Construct a chromatogram and calculate elution volume as the time from the start point to the apex of the peak for the protein (or other solute) multiplied by the flow rate of the pump (as determined in step 16).

    If large sample volumes are being applied, the correct elution volume of the peak is obtained by subtracting half the applied sample volume from the apparent elution volume.

    For determination of void volume as in step 17, the fractions that emerge between the point where a volume of buffer equal to 25% of the bed volume has passed through the column and the point where buffer volume equal to 50% of the bed volume has passed through the column are analyzed (either in line by monitoring detector response or off line by another method; see annotation below). For determination of the total liquid volume as in step 18, fractions emerging between the point where a buffer volume equal to 75% of the bed volume has passed through and that where 125% has passed through are analyzed. To determine the elution volume of an unknown protein sample (as in determining molecular size; see Basic Protocol 3), fractions emerging between the point where a buffer volume equal to 25% of the bed volume has passed and that where 125% has passed through are analyzed. Allowing 125% to pass through will permit the entire peak to be traced.

    If fractions are to be collected for subsequent off-line analysis to determine where elution occurs (e.g., by photometry at 280 nm to detect Blue Dextran 2000 or proteins, or conductivity to trace the salt peak), start and stop the fraction collector to sample fractions at the volumes stated above. The fraction size should be large enough to permit accurate assay of the solute of interest; often 1 ml or 2% of the bed volume (whichever is smallest) is a suitable fraction size. The prefraction (i.e., the amount of liquid eluted from the point of sample application to the start of fraction collection for analysis) should be sampled in a vessel of known weight. The elution volume is calculated from the volume of the fractions by adding half the volume of the peak fraction and the volume of the prefraction, then subtracting half the sample volume. For dilute aqueous buffers (e.g., 0.5 M) a density of

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1 may be assumed for converting weight to volume. If the experiment will take a long period of time, it is advisable to minimize evaporation by sealing the fraction cups with Parafilm.

If an open-bed (gravity flow) column is used, carefully add a volume of GF buffer that corresponds to the void volume of the column reduced by half the sample volume—unless the sample volume is <4% of the bed volume, in which case add 1 void volume of buffer. If the column is operating correctly, the effluent collected should contain no protein. Add GF buffer in ≤1-ml increments if the column has a bed volume <50 ml, collect fractions, assay for proteins, and calculate elution volumes as described above.

23b. *To desalt a protein:* Pass a volume of GF buffer equal to the void volume reduced by half the applied sample volume through the column, and collect a waste fraction. Place a vessel that can hold 1.5 times the applied sample volume under the column, then add a volume of GF buffer equal to the applied sample volume. Collect the desalted protein.

Depending upon the efficiency of the desalting system, these quantities may have to be adjusted (i.e., the volume collected in the second fraction may need to be decreased in order to preclude contamination from substances that elute later). If system effects (e.g., mixing after the column) are substantial or the column is badly packed (e.g., contains cracks in the bed), it will be necessary to pass more GF buffer through the column than the void volume minus half the sample volume before sampling the protein (see Critical Parameters). Figure 8.3.3 shows a sample chromatogram of optimized desalting of 400 ml hemoglobin.

The desalting step may be continuously monitored by simultaneous in-line detection of the protein zone by absorbance at 280 nm and in-line detection of the salt peak by conductivity.

24. Wash column with ≥1 column volume of GF buffer containing an antibacterial agent. Close column outlet and store column.

See recipe for GF buffer for acceptable antibacterial agents.

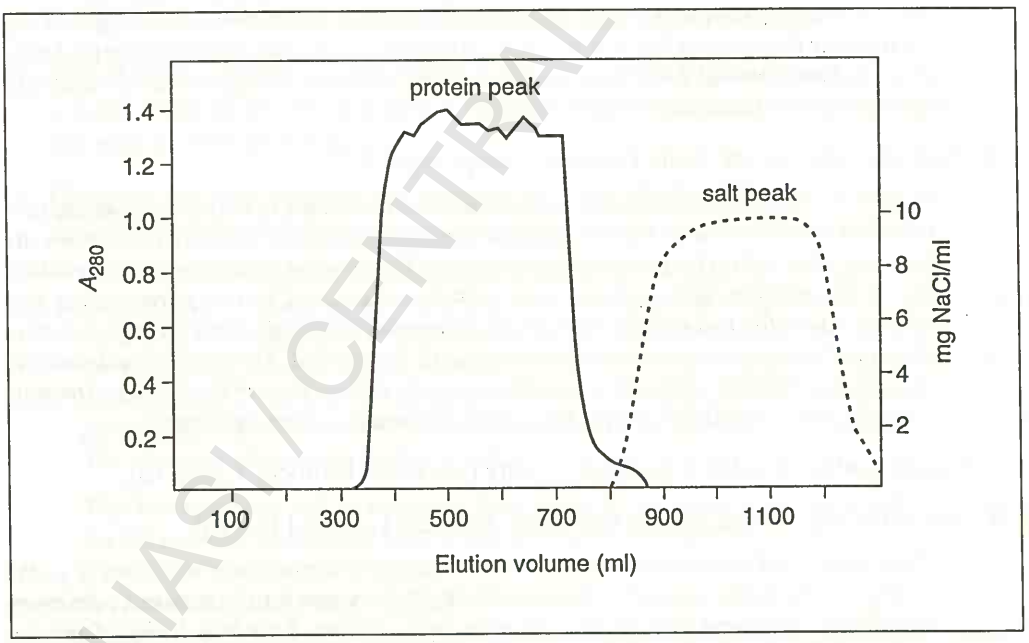

Figure 8.3.3 Chromatogram illustrating desalting of proteins by gel filtration. A 4 × 85–cm column packed with Sephadex G-25 is used. The sample consists of 400 ml hemoglobin (protein peak; solid line) in NaCl (salt peak; broken line). Note that the sample volume is close to the pore volume of the packed matrix, i.e., 490 ml. No correction for sample volume has been made. Calculation from the figure yields V_o = 560 ml – 200 ml = 360 ml, which is 31% of the geometric column volume (V_c). Reproduced from Flodin (1961) with permission of the *Journal of Chromatography*.

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PROTEIN FRACTIONATION

Protein fractionation refers to the separation of proteins of similar molecular size for purification purposes. The objective may be to separate dimer and higher aggregates from a pharmaceutically active protein or peptide. Requirements for the column and system are therefore substantially higher for protein fractionation than for desalting.

The same materials and methodology described for desalting are used for protein fractionation. However, selection of matrix and column (see Strategic Planning) is more critical in this procedure, as are the quality of the column packing and control over experimental conditions (e.g., temperature and flow rate). The protocol includes steps for determining the column efficiency (also see *UNIT 8.1*) to evaluate the fitness of a packed column for use in protein fractionation.

Materials

GF matrix (see Table 8.3.4) or prepacked GF column (see Table 8.3.6) with appropriate selectivity for protein of interest (also see Strategic Planning and see Critical Parameters)
GF fractionation buffer (see recipe)
Colored marker: 0.2 mg/ml Blue Dextran 2000 or 0.2 mg/ml vitamin B_{12}
Low-molecular-weight marker (e.g., 5 mg/ml acetone or 2 M sodium chloride)
Protein sample to be fractionated
GF chromatography column (see Critical Parameters and Table 8.3.5)

Prepare column

1. Prepare GF matrix as described in the desalting procedure (see Basic Protocol 1, steps 1a to 3a or 1b to 3b) using GF fractionation buffer wherever GF buffer is indicated.

   It is generally difficult to prepare columns of maximum efficiency using a GF medium with a small (e.g., 10-μm) particle size. The need to scale up the procedure should be considered when selecting medium and column (see Strategic Planning and Critical Parameters).

   The GF fractionation buffer must be selected in light of the method planned for testing purity of the final protein fraction (step 11), as this may put special restrictions on the buffer (e.g., sodium azide absorbs light at 254 nm and will also interfere with the anthrone reaction used for assaying carbohydrates).

2. Pack the column (see Basic Protocol 1, steps 4 to 12).

   Because protein fractionation puts high demands on the quality of the packed column, care must be taken in selecting columns of proper design. Columns for protein fractionation are typically 30 to 100 cm long, depending upon the difficulty of the separation and the particle size of the medium used. Columns used must be constructed so that parts coming into contact with buffer and sample are made of appropriate materials. Long tubings and other sources of dead space or mixing chambers should be avoided. The pressure resistance of the column should be compatible with the expected pressure drop of the bed. See Strategic Planning and Critical Parameters for details concerning column selection.

3. Check quality of column packing visually (see Basic Protocol 1, step 13).

4. Assemble and test the system (see Basic Protocol 1, steps 14 to 16).

   Place system and column in an environment not subject to temperature variations (e.g., the column should not be exposed to direct sunlight). If the system is to be used in a cold room, the influence of temperature on viscosity must be considered. Zone broadening of proteins and the pressure drop of the system are proportional to the viscosity of the buffer, and the flow rate will have to be adjusted accordingly.

   Successful, reproducible protein fractionation requires a constant flow rate. This may be achieved using a peristaltic or high-precision pump whose flow rate is regularly checked. Continuous monitoring of the effluent is necessary to ensure proper sampling of the fraction

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of interest. Detection may be carried out at 280 or 254 nm to trace proteins containing aromatic amino acids, or at lower wavelength (e.g., 214 or 206 nm) to measure absorption by peptide bonds. Other parts of a protein fractionation system that might be of use include a fraction collector (although in the simplest case it is possible to collect the fraction of interest by using a switch valve) and a chromatography controller, which permits automatic fractionation of the protein of interest.

Evaluate column

5. Chromatograph a colored marker (2 mg/ml Blue Dextran 2000 or 0.2 mg/ml vitamin B_{12}; see Basic Protocol 1, steps 20 to 23) and ascertain that the zone produced is horizontal and sharp.

   A volume of marker solution 1% to 4% of the bed volume should be applied. The zone produced by a colored sample must be horizontal and sharp for packing to be considered acceptable.

   If the supplier recommends a different method than this for evaluating column packing, that method should ordinarily be used.

6. Chromatograph a low-molecular-weight marker (e.g, 5 mg/ml acetone or 2 M sodium chloride; see Basic Protocol 1, steps 20 to 23) and construct a chromatogram to determine the column efficiency.

   The sample volume applied in this step may contribute considerably to the peak width and result in a falsely low value for column efficiency. It is therefore recommended that the sample volume not exceed 0.9%, 0.5%, and 0.4% of the bed volume for columns packed with GF matrices having average particle diameter of 100 μm, 30 μm, and 10 μm respectively (Hagel, 1985).

7. Calculate the number of theoretical plates per column according to the equation $N = 5.54(V_r/W_h)^2$, where N = number of theoretical plates per column, V_r = elution (retention) volume of peak, and W_h = width of the peak at half peak height. See Figure 8.3.2 for a graphical depiction of these variables.

   The efficiency of the column corresponds to the number of theoretical plates under given experimental conditions. See UNIT 8.1 for discussion of column efficacy.

8. Calculate the asymmetry factor of the peak according to the equation $A_s = (b/a)$, where a is the width of the leading part of the peak and b is the width of the tailing part of the peak at 10% peak height.

9. Compare the plate number and asymmetry factor obtained for the column with the acceptance limits for these parameters in the manufacturer's documentation.

   If no data for expected column performance is given, calculate the plate height (H) according to the equation $H = L/N$, where L is the length of the packed bed and N is the number of theoretical plates calculated in step 7. As a rule of thumb, H should be about three times the average particle diameter of the matrix for a good column and twice the average particle diameter for an excellent column. Plate heights of five times the average particle diameter or more are generally not accepted for high-resolution chromatography. The asymmetry factor should be 1.0 ± 0.2.

   The flow rate used will influence the broadening of the solute peak; suggested flow rates for GF matrices of different particle size are given in Table 8.3.7.

Perform chromatography and evaluate results

10. Dissolve, apply, and chromatograph protein sample to be fractionated (see Basic Protocol 1, steps 20 to 23).

    Start by applying a small fraction of the sample to get a rough idea of the separation and to calculate optimum conditions for the actual run. A sample volume of ~1% to 4% of the bed volume has been found to be optimal for protein fractionation using a medium of 30-μm

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particle size, depending upon the processing rate (i.e., amount of substance processed per unit time) of the system (Hagel et al., 1989). As a general rule, a sample volume of 2% of the bed volume can be applied. A chromatogram illustrating protein fractionation where dimers and higher aggregates are separated from the target monomeric form of the protein is given in Figure 8.3.4.

Optimum sample volume depends upon many experimental factors; discussion of optimization strategies have been published (Hagel et al., 1989; Hagel and Janson, 1992). In general, the larger the bead size used, the larger the sample volume that may be applied before the initial resolution is lost. This is due to the fact that smaller bead sizes normally result in more efficient columns that produce narrower peaks; hence the influence of sample volume on peak width is more detrimental.

Table 8.3.7 Practical Velocities for Protein Fractionation or Size Determination[a]

Solute molecular mass	Nominal eluant velocities (cm/hr) at a support particle size of (μm):						
	2	5	10	15	30	50	100
1,000	507	203	101	68	34	20	10
5,000	293	117	59	39	20	12	6
10,000	234	94	47	31	16	9	5
15,000	195	78	39	26	13	8	4
50,000	137	55	27	18	9	6	3
75,000	121	48	24	16	8	5	2
100,000	109	44	22	15	7	4	2
200,000	86	34	17	11	6	3	2
300,000	76	30	15	10	5	3	2

[a]Adapted from Hagel (1989) with permission from VCH Publishers.

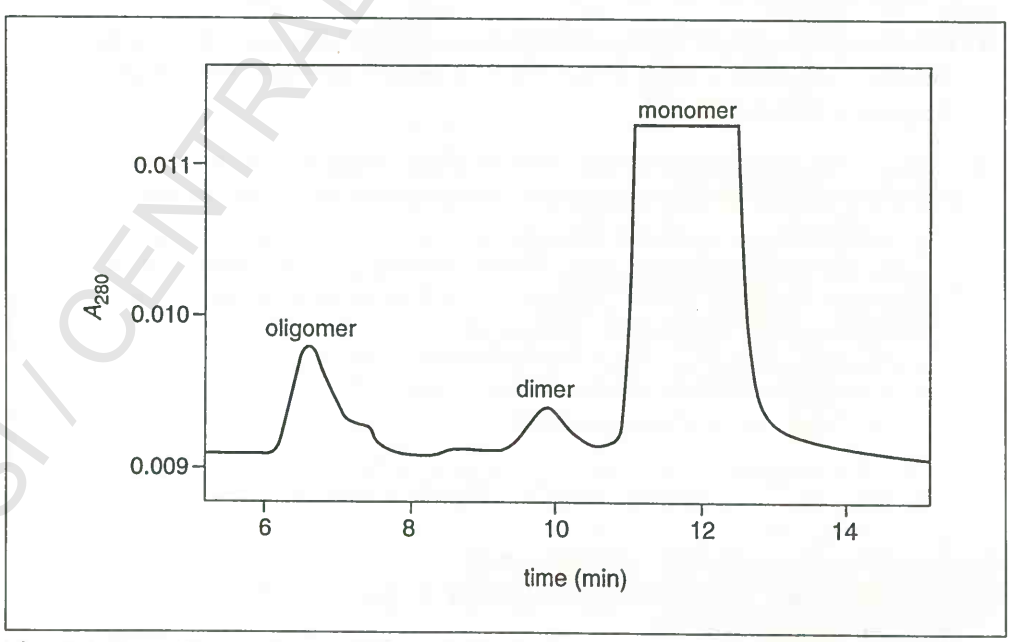

Figure 8.3.4 Chromatogram illustrating protein fractionation by gel filtration. A Superdex 75 HR 10/30 column is used. The sample consists of recombinant human growth hormone. This figure is an enlarged section of the original chromatogram displayed to illustrate complete resolution between monomer and dimer. Reproduced from Hagel (1993) with permission of the publisher. Courtesy of B. Pavlu, Kabi-Pharmacia Peptide Hormones, and H. Lundström, Pharmacia Biotech.

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11. Check the purity of the collected fraction(s).

Suitable methods of determining purity include HPLC and electrophoresis (see UNITS 10.1-10.4).

DETERMINATION OF MOLECULAR SIZE

BASIC PROTOCOL 3

The third important application of gel filtration is in determining molecular size. To accomplish this, a GF column is calibrated with reference samples of known molecular size (see Support Protocol). Gel filtration is then carried out on an unknown protein solute as in Basic Protocols 1 and 2, and the molecular size of that solute is then inferred from the calibration curve, which relates elution volumes or distribution coefficients on the calibrated column to the molecular sizes of the different reference samples. If the relationship between the molecular size and mass is known, the mass of the solute may be calculated. However, because the shapes of biological molecules vary considerably (e.g., proteins may be spherical or ellipsoidal, or in some cases more or less rod-shaped), the calculation of molecular mass (M_r) from elution volume must be done with great care. If the shape of the molecule under study is not known, the experiment will yield only its estimated solvated size. The influence of solute shape on size may be exemplified by the fact that the radius of gyration (one estimate of size) is proportional to $M_r^{1/3}$ for spherical molecules (e.g., some proteins), to $M_r^{1/2}$ for molecules shaped like flexible coils (e.g., dextrans and denatured proteins), and to M_r for rod-shaped molecules (e.g., fibrous proteins and DNA fragments of intermediate length). Thus, gel filtration is very suitable for estimating the molecular size but generally not the molecular mass of unknown solutes.

Materials

GF matrix or prepacked GF column (see Table 8.3.4 and Table 8.3.6; see Strategic Planning; see Critical Parameters)
GF fractionation buffer (see recipe; note variations for size determination)
GF chromatography column (Table 8.3.5)
High-precision pump
Protein sample for size determination

1. Prepare gel, pack column, then assemble and test GF system (see Basic Protocol 1, steps 1 to 16) using GF fractionation buffer wherever GF buffer is indicated.

For high-precision analytical gel filtration, the temperature of the chromatographic bed is often kept constant by using a jacketed column and circulating water from a temperature-controlled water bath. This makes the instrument setup more complicated, and the need for this precaution must be assessed on a case-by-case basis (e.g., if the column is calibrated at every other run, as is possible with high-speed gel filtration, there is little need for temperature control).

A high-precision pump must be used to ensure accurate and constant flow rate, which is of utmost importance for achieving reliable results in molecular size determinations. In addition to a UV detector for determining protein concentration, a detector for viscosity and/or light scattering—e.g., one employing multiple-angle laser light scattering (MALLS)—may be used for assay of molecular size or (indirectly) molecular mass. A mass spectrometry detector—e.g., one employing electrospray ionization mass spectrometry (ESI-MS)—may be used for assay of molecular mass.

If denatured standards are to be used in the calibration to eliminate the influence of molecular shape (see Support Protocol), 6 M guanidine hydrochloride (see recipe for GF buffers in Reagents and Solutions) is used as the GF fractionation buffer in this and subsequent steps.

Once a column has been calibrated (see Support Protocol), it is important to check column performance by determining the elution volume of one of the reference samples used for calibration as part of the routine testing of the system.

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2. Determine the void volume (V_o) and the total liquid volume (V_t) for the system (see Basic Protocol 1, steps 17 and 18).

3. Calibrate column (see Support Protocol). Check flow rate during calibration by sampling effluent and weighing fractions.

   Calibration of the column (and chromatography of the sample) may be carried out under denaturing conditions to eliminate the effects of molecular shape. Variations in procedure are described in the Support Protocol.

   There are no general restrictions on the flow rate as long as the protein of interest separates from contaminating solutes (ideally, baseline separation should be achieved). A high flow rate will not influence the qualitative information sought—i.e., the position of the peak maximum. Thus, qualitative information may be obtained by very fast gel filtration; however, at high flow rates the danger of shear effects on elongated solutes must be considered. If a high-precision pump is used, there is less need to check the flow rate for every run (though in the author's experience this is a good practice because any pump may malfunction). If constant flow rate can be taken for granted, time may be substituted for volume in the calculations.

   For both the calibration (see Support Protocol) and the run to determine molecular size of the unknown sample, the elution volume should generally be corrected for the contribution from the sample volume (see Basic Protocol 1, step 23a). However, if the sample volume is kept constant throughout the entire investigation it may arbitrarily be regarded as a part of the dead volume of the system, and no correction is necessary.

4. Apply, elute, and chromatograph unknown sample (see Basic Protocol 1, steps 20 to 23). Prepare a chromatogram and calculate elution volume. Check flow rate by sampling the effluent and weighing fractions.

   The same applied sample volume should be used for the unknown sample as for the size standards (see Support Protocol).

   If denatured standards were used in the calibration to eliminate the effects of molecular shape (see Support Protocol), the sample must be denatured. Elution is then carried out using 6 M guanidine hydrochloride as the GF buffer.

   Often the calculations are performed on a time basis using an integrator or chromatography software (Amersham Pharmacia Biotech).

5. Calculate molecular size of unknown sample components using the calibration graph constructed in performing the support protocol.

   If two different detectors were used for the reference standards and the sample (e.g., a refractive index detector for calibration with dextran standards and a UV detector for the protein sample), a correction factor for the difference in volume between the two detectors must be used in the calculations.

   The estimate of molecular size will be correct only if there are no sample-matrix or sample-sample interactions (in fact, gel filtration has been used to study these self-association processes). The size estimate will be an apparent gel-filtration size which, together with data about the molecular mass (e.g., from mass spectrometry), will yield information about molecular shape.

6. Continue to run GF buffer through column until the next run is to be made.

   It is important to continue flushing the column to maintain column characteristics and avoid the need for recalibration (see Critical Parameters). If the next run is not to be made for a long time, the flow rate may be reduced and buffer recirculated through a filter to the buffer reservoir (see Critical Parameters). Before reusing the column after a long period of rest, the validity of the calibration curve should be checked by running two or three reference samples covering the range of interest and calculating their molecular size from the old calibration curve. The apparent size should agree with the assigned size within ±5% error.

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COLUMN CALIBRATION

Molecular size standards for column calibration should ideally be of the same shape and type as the molecules under study (e.g., globular proteins, fibrous proteins, or peptides). In cases where this requirement cannot be met, the column can be calibrated with other suitable reference substances of known size (e.g., dextrans). An apparent size may thus be assigned to the unknown and the actual size then inferred from the relationship between the shape of the different molecules. It is thus possible to use dextran as a reference for the estimation of Stokes radius for globular proteins (Hagel, 1993). However, a general shape parameter governing separation of all types of molecules by gel filtration has not yet been found (Dubin et al., 1990).

When protein standards are used, the calibration curve will reflect small natural variations in the molecular shapes of proteins, which will decrease the precision of the curve. However, the procedure described here is very simple and will readily yield an apparent molecular mass or molecular weight, which in many cases is quite satisfactory.

The influence of protein shape on the calibration curve may be eliminated by disruption of noncovalent bonds with denaturing solvents (e.g., guanidine hydrochloride). Disulfide bonds are broken by reductive cleavage with a reagent such as DTT, and reoxidation is prevented by carboxymethylation. This treatment converts the shape of sample and reference proteins to random coils. The physical size of a random coil is larger than that of a compact molecule of equal mass; therefore a more porous gel is needed to separate the former species. Detergents may also be used to denature proteins. However, it must be noted that a protein-detergent complex is larger than the protein and that ionic detergents will introduce charged groups. Also, information about size or molecular mass obtained using detergent-denatured proteins may not be relevant to the native protein. As these types of treatment are frequently used to prepare proteins for analytical electrophoresis, the reader is referred to *UNITS 10.1-10.4* for further details.

The influence of variation in shape on the calibration curve may also be eliminated by using as molecular size standards reference substances from a homologous class of nonprotein polymers—e.g., dextran or polyethylene glycol. Dextran has been used extensively as reference substance for calibration of gel-filtration columns. Calibration can be done by running a number of narrow fractions or one sample of broad size distribution.

***Additional Materials** (also see Basic Protocol 3)*

Calibration standards (see recipe and Table 8.3.8)
6 M guanidine hydrochloride (*APPENDIX 3A*)
In-line refractive-index detector

For calibration with native proteins

1a. Calibrate the column using protein standards (see Table 8.3.8) as in steps 20 to 23 of Basic Protocol 1 and construct a chromatogram.

It is practical to mix a number of the calibration standards and chromatograph them simultaneously so that the calibration may be completed in a few runs (see example in Figure 8.3.5).

2a. Plot the elution volume (V_r; i.e., the volume on the chromatogram from the injection point to the apex of the peak for a particular size standard) versus the logarithm of the molecular size, $\log_{10}R$, where R is the molecular radius of the calibration standard.

Table 8.3.8 gives the molecular radii of commonly used calibration standards. A sigmoid curve should be obtained that is approximated by a straight line in the middle section (see Fig. 8.3.5).

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If the sample volumes are not held constant, the elution volume must be normalized by setting the injection point at half injected volume.

The calibration curve is often made system-independent by plotting distribution coefficient (K_D) versus $\log_{10}R$. The distribution coefficient is calculated as

$$K_D = (V_r - V_o)/(V_t - V_o),$$

where V_r is the elution volume for a particular peak, V_o is the void volume for the column, and V_t is the total liquid volume for the column.

For substances belonging to a homologous series (e.g., dextrans), it is meaningful to plot elution volume versus the logarithm of the molecular mass ($\log_{10}M_r$). Where substances of different chemical nature are being used to calibrate the column, the preferable way to

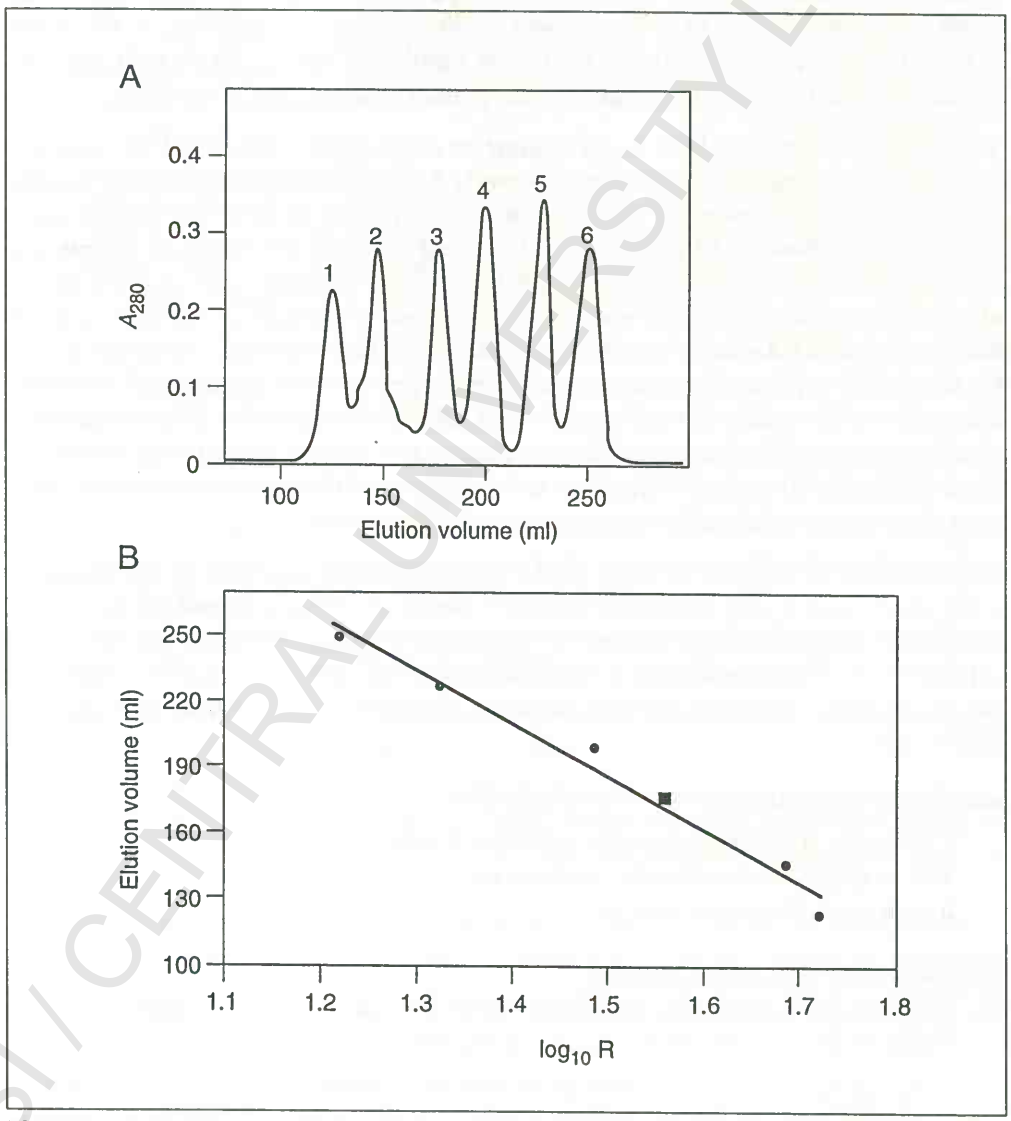

Figure 8.3.5 Calibration of GF column for determination of protein size. A K 26/70 column packed with Sephadex G-200 SF is used. **(A)** Chromatogram obtained for six size standards: 1, catalase; 2, aldolase; 3, bovine serum albumin; 4, ovalbumin; 5, chymotrypsinogen A; 6, ribonuclease A. **(B)** Calibration curve obtained by plotting the logarithms of the molecular radii ($\log_{10}$R) of the standards (*x* axis) versus the elution volumes for peaks 1, 2, 4, 5, and 6 from panel A (filled circles). The curve was used for evaluating the size of bovine serum albumin (filled square; elution volume corresponds to peak 3 in panel A), giving an apparent size of 34.8 Å. The excellent agreement with the nominal molecular size value of 35.5 Å may be incidental. Repeating the procedure for peak 4 in panel A showed that a deviation of ~3 Å from the nominal value may be expected for proteins of globular shape. Panel A reproduced from Pharmacia Biotech (1991) with permission from the publisher.

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calibrate the column is by using the hydrodynamic volume, V_h. This is calculated according to the equation $V_h = (\eta)(M_r)/N\nu$, where η is the intrinsic viscosity, M_r is the relative molecular mass, N is Avogadro's number (6.02×10^{23} molecules per mole), and ν is a shape factor, which for a spherical solute is 2.5.

It is wise to check the accuracy of the calibration by deleting one point at a time from the calibration data set and calculating the size or mass of the protein represented by that point from the calibration curve obtained with the remaining data. The average absolute difference from the nominal value indicates the accuracy of the calibration (see Fig. 8.3.5).

For calibration with denatured standards

1b. Denature calibration standards according to a standard denaturing protocol.

2b. Carry out steps 1a and 2a above, using a column equilibrated in 6 M guanidine hydrochloride and using 6 M guanidine hydrochloride as the GF buffer in the cross-referenced steps of Basic Protocol 1.

Where denatured standards are used, denaturing conditions must be maintained in chromatography of the sample (see Basic Protocol 3).

For calibration with nonprotein polymer standards

1c. Using an in-line refractive index detector, run system until a stable baseline is obtained. Chromatograph a number of dextran calibration standards (see Basic Protocol 1, steps 20 to 23) that will give calibration points surrounding the protein under study, to obtain their elution volumes or distribution coefficients.

The proper number and size of calibration standards to be run may be quickly found by running first the unknown sample and then the necessary calibration samples that will be eluted close to the unknown protein.

Apply each standard individually or as a mixture of two dextrans of high and low molecular mass.

Using a two-channel recorder set at two different sensitivities may prevent the peak from running off scale.

2c. Plot the elution volume (V_r) or distribution coefficient (K_D) versus the logarithm of the viscosity radius of dextran ($\log_{10} R_{vis}$).

The viscosity radius of dextran, R_{vis}, is related to the molecular mass (M) according to the equation $R_{vis} = 0.271 \times M^{0.498}$. Because dextran is a polydisperse polymer, the value of M used here is the molecular mass of dextran that corresponds to the peak apex, sometimes denoted M_p.

When two different detectors are used for calibration and chromatography of the sample—e.g., a refractive index detector for dextran standards and a UV detector for the protein sample—the difference in volume between the two detectors must be taken into account in calculation of elution volume.

Another procedure where the entire separation range of interest was calibrated in one run, using an integral calibration method, has been described (Hagel and Andersson, 1991; Hagel, 1993). Unfortunately, this simple and powerful method cannot yet be performed on routine basis because it requires data for the molecular mass distribution of the sample, and this information is, at the moment, not readily available in most cases.

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REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Calibration standards

Table 8.3.8 provides a compilation of commercially available molecular size calibration standards and their suppliers. Some of these are available as kits (e.g., from Amersham Pharmacia Biotech). Protein standards should be prepared in the GF buffer that will be used in the molecular size determination. To prolong the life of the column, samples should be filtered through a protein-compatible filter prior to injection. The exact concentration to be prepared depends on the additives present (e.g., sucrose as stabilizer), detector sensitivity, and the zone broadening of the column. Concentrations of proteins in the range from 1 to 5 mg/ml are frequently used for calibration. It is possible to mix several standards to calibrate the separation range of interest in a few runs (it is seldom necessary to calibrate the entire separation range of the column).

Table 8.3.8 also lists some dextran fractions that are useful for calibrating the column in hydrodynamic volume, which makes comparing solutes of different shape easier.

GF buffers

For desalting: Use a volatile buffer—e.g., 0.05 M ammonium hydroxide, 0.05 M acetic acid, 0.05 M ammonium acetate, or 0.5 M ammonium bicarbonate—unless the step is for buffer exchange, in which case use the desired final buffer.

Distilled (Milli-Q-purified or equivalent) water may be preferred to buffer as an eluant in some cases. This will yield adequate results with noncharged solutes; however, ionic interactions with the small amount of charged groups on the gel surface may disturb the purification of slightly charged molecules when water alone is used. Addition of a volatile bacteriostatic agent—e.g., chlorhexidine—may sometimes provide enough ionic strength to suppress such interactions.

For protein fractionation: Use 0.05 M sodium or potassium phosphate (pK_a = 2.1, 7.2, 12.4), 0.05 M Tris·Cl (pK_a = 8.1), or 0.05 M sodium or potassium acetate (pK_a = 4.8).

The buffering capacity of these buffers is adequate at a pH within ±0.5 units of the pK_a value. If the protein is to be freeze-dried, use of a volatile buffer or water is advantageous.

It is sometimes necessary to add 0.1 M sodium chloride to completely eliminate ionic interactions. If parts of the system that come into contact with the buffer are composed of steel, the sodium chloride should be replaced with 0.05 M sodium sulfate to prevent halide-induced corrosion. In some cases the ionic strength of the buffer will affect the sample, e.g., by disrupting aggregates. This effect can be eliminated only by changing the ionic strength.

For determining molecular size: Use the same buffers as for protein fractionation but select one that will preclude solute-matrix interactions (see Support Protocol). If the column is to be used for a long period, add preservatives to prevent bacterial growth. Make sure the buffer is compatible with the detection step (e.g., use a buffer with low salt content if mass spectrometry will be used for detection).

If calibration is to be conducted under denaturing conditions, 6 M guanidine hydrochloride is used as the GF buffer. Guanidine hydrochloride (APPENDIX 3A) is a better denaturing agent than urea.

For all buffers: Add antimicrobial agent if buffer will be used for a long time, adjust the pH of the buffer, filter through a 0.22-µm filter, then recheck pH and readjust if necessary.

It is also recommended to degas the buffer prior to prolonged use. It is common practice to prepare fresh buffer once a week, but if an antimicrobial agent has been added it may be

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stored for 1 month or more. Antimicrobial agents used for GF buffers include ethanol added to a concentration of 20% (v/v), sodium azide added to a concentration of 0.02% to 0.05% (w/v), or chlorhexidine added to a concentration of 0.002% (w/v). Ethanol is not recommended for use with Sephadex gels as organic solvents cause the bed to shrink when used with this matrix.

IMPORTANT NOTE: Sodium azide is a very potent antimicrobial agent and therefore widely used. However, handling of azide, especially the dry powder, requires special attention; please refer to information from the supplier for safety precautions. Frequent exposure to the dry powder may be avoided by preparing a stock solution (e.g., 20% w/v) from which aliquots (e.g., 1 ml azide stock solution to 1 liter GF buffer) may be drawn conveniently with a micropipet. Azide may form explosive salts in lead-pipe waste disposal systems; collect the effluent in waste bottles.

Total liquid volume marker

5 mg/ml acetone, 10 mg/ml sodium azide, 0.1 mg/ml cytidine, 10 mg/ml sodium nitrate, and 2 M sodium chloride have been used for determination of total volume. A negative pulse from injection of water can also be used. In cases where interactions between matrix and solute must be eliminated, deuterium oxide can be used.

Acetone and cytidine are detected by UV absorbance at 280 nm, azide at 254 nm, and nitrate at 254 nm. Sodium chloride can be detected by flame photometry, conductivity, a sodium or chloride electrode, or precipitation of chloride with silver nitrate solution. A negative pulse from injection of water is detected by measuring the absorbance or refractive index of the buffer or by monitoring the conductivity of the effluent. Deuterium oxide is detected with a refractive-index detector.

Void marker

A suitable substance for determining void volume is a protein of the correct size (not too large and not too small), which may be selected by running a series of large proteins and observing which of them start to permeate the matrix. Other substances that have been used include 2 mg/ml Blue Dextran 2000 and DNA.

Blue Dextran 2000 has the advantage of being visible to the eye. Disadvantages are that small amounts of free dye may stick to the gel and a broad permeating peak may be visible in addition to the sharp excluded peak if the pore size of the gel is large enough (e.g., as with Sepharose 4B). This occurs because of the polydispersity of dextran. If the molecule is too large, so-called secondary exclusion effects, in which the sample is excluded from the narrow space where beads intersect, will yield a falsely low void volume. However, this phenomenon is only important in high-precision gel filtration and rarely has to be considered in desalting.

COMMENTARY

Background Information

Although separation of molecules according to size had been noticed in the mid-1950s (Lindqvist and Storgårds, 1955; Lathe and Ruthven, 1956), the discovery of gel filtration (GF) may be attributed to Porath and Flodin (1959), who realized the potential of the technique and were the first to report using it to desalt proteins. Their discovery provided the basis for a new separation technology that rapidly gained widespread acceptance owing to the commercial availability of a suitable cross-linked gel-filtration matrix—Sephadex—introduced in 1959. Remarkably enough, after 35 years this matrix is still one of the premier choices for desalting of proteins (see Table 8.3.1). The use of gel filtration for determination of molecular size of proteins was described later (Andrews, 1970). Gel filtration was also rapidly established as an important tool for determining the molecular mass distribution of polymers in aqueous (Granath and Flodin, 1961) and organic solutions (Moore, 1964), for which suitable methods were lacking at that time. Moore used cross-linked polystyrene-based media for his application, naming the technique gel-permeation chromatography. Thus, within a few years after the discovery of gel filtration, applications of the technique basically covered the present range.

During the following years, the focus was directed to investigating the mechanism behind gel filtration. Theories suggesting that the separation was regulated exclusively by diffusion

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of molecules were soon replaced by the explanation, accepted today, that the process is driven by loss of conformational entropy when free permeation of the matrix by a solute is hindered by steric factors (Casassa, 1967). Of course, diffusion affects peak width and thus also the degree of separation, but diffusion does not influence the elution volume of a solute. The definite impact of steric exclusion on the separation has led to the proposal to rename the technique "steric exclusion chromatography" (ASTM, 1978), but this term has not gained wide acceptance. Another generic designation sometimes used in the literature is size-exclusion chromatography (SEC). It is important to realize that all of these designations refer to the same basic separation principle, which the majority of protein chemists refer to as gel filtration.

Development of gel-filtration media was focused for a long period on natural polymers or hydrophilic polymers. The late 1970s saw the introduction of silica-based materials, which combined rigidity and small particle size to enable fast gel filtration. A few years later, natural polymers of enhanced rigidity were

Table 8.3.8 Molecular Size Standards for Calibration of Gel Filtration Columns

Standard	Molecular mass (M_r)	$\log_{10}(M_r)$	Molecular size (radius, Å)[a]	Supplier[a]
Proteins[b]				
Thyroglobulin, bovine thyroid	669,000	5.825	85.0	PB
Ferritin, horse spleen	440,000	5.643	61.0	PB
Catalase, bovine liver	232,000	5.365	52.2	PB
Gamma globulin, bovine	158,000	5.199	—	BR
Aldolase, rabbit muscle	158,000	5.199	48.1	PB
Transferrin	81,000	4.908		SI
Albumin, bovine serum	67,000	4.826	35.5	PB
Ovalbumin, chicken	44,000	4.643	—	BR
Ovalbumin, hen egg	43,000	4.633	30.5	PB
Chymotrypsinogen A, bovine pancreas	25,000	4.398	20.9	PB
Myoglobin, equine	17,500	4.243	—	BR
Ribonuclease A, bovine pancreas	13,700	4.137	16.4	PB
Non-protein standard				
Vitamin B_{12}	1,350	3.130	—	BR
Dextrans[c]				
Dextran T-10	8,100		23	PB
Dextran T-40	23,600		40	PB
Dextran T-70	33,000		50	PB
Dextran T-500	370,000		150	PB
Dextran 1	1,080		9	PC
Dextran 5	4,440		18	PC
Dextran 12	9,980		26	PC
Dextran 25	21,400		39	PC
Dextran 50	43,500		55.3	PC
Dextran 80	66,700		68.4	PC
Dextran 150	123,600		93.1	PC

[a]Abbreviations and symbols: —, not stated by manufacturer; BR, Bio-Rad; PB, Amersham Pharmacia Biotech; PC, Pharmacosmos; SI, Sigma. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

[b]The size of proteins may vary according to the source of information and the methodology used for determination—e.g., a value of 67.1 Å has also been assigned to ferritin (de Haen, 1987).

[c]Dextran is a polydisperse polymer and values given here—e.g., peak molecular mass—may vary from lot to lot. In cases where peak molecular mass is not stated by the supplier, the column is calibrated as elution volume versus $M = (M_w \cdot M_n)^{1/2}$, where M_w is the weight-average molecular mass and M_n is the average molecular mass. The molecular size of dextran is calculated according to the equation $R_{vis} = 0.271 \times M^{0.498}$, where R_{vis} is the viscosity radius and M is the molecular mass (Hagel, 1989).

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introduced, which made it possible to pack columns with matrices of 10-μm bead size. To distinguish these media from the traditional type, the designation of high-performance size-exclusion chromatography (HPSEC) was introduced. However, it has been recently pointed out that the advantage of HPSEC media lies not in performance (i.e., traditional media packed in long columns may have equal or even better resolving capacity) but in speed of operation (Hagel, 1992). In addition, none of these materials have the outstanding selectivity shown by some of the traditional matrices (e.g., Sephadex and Bio-Gel P). Therefore, development of matrices for gel filtration has now been directed toward increasing their selectivity, which is done by decreasing the apparent width of the pore-size distribution of the matrix. Development is also geared toward tailoring the apparent pore size of matrices for the separation of molecules in specific interesting areas of application (e.g., for polishing of recombinant peptides). Recently, some of these modern media have become available for separation of peptides and proteins of moderate size (Hagel, 1993).

Even though the theoretical framework for the separation mechanism in gel filtration is well understood, there are still some uncertainties as to which molecular size estimate the elution volume reflects (Dubin et al., 1990). It is generally agreed that Stokes radius is a poor measure of molecular size, especially if different types of molecules are being studied. The hydrodynamic volume seems to be a general size parameter for polymers, globular proteins, and DNA of a certain length (Potschka, 1987). It appears that more work needs to be done to resolve this fundamental issue.

Until recently, development of instrumentation for gel filtration has been modest. High-precision pumps and automated systems for injection, fraction collection, and calculation were introduced more than a decade ago. New equipment and technology being introduced includes multiple-angle laser light scattering (MALLS) detectors and in-line viscosity detectors, which provide information about the shape and size of molecules, and electrospray ionization mass spectrometry (ESI-MS), which has the power to provide in-line detection of molecular mass. ESI-MS combined with high-resolution gel-filtration columns provides a very powerful system for characterizing protein dimensions. Molecular size of solutes may also be determined with an in-line light-scattering or viscosity detector combined with a concentration-sensitive detector. It is now possible to obtain the exact molecular mass of small-to-medium-sized proteins by connecting an in-line mass spectrometer to the GF column. The advantages of using a mass-sensitive detector are that the system does not need to be calibrated, fluctuations in flow rate are not detrimental, and solute-matrix interactions or solute-solute interactions will not affect the molecular mass obtained. In this case, the column acts as a high-resolution separating device to resolve the sample components prior to determination of molecular mass by mass spectrometry.

Critical Parameters

Optimization and scaleup of desalting

Optimization of desalting starts with selection of a matrix of suitable exclusion limit with maximum pore volume and proper particle size. The bed volume is dictated by the sample volume to be purified. The actual elution protocol to be used depends upon the dispersion of the chromatography system, which in turn is related to the particle size and the column length. The particle size will influence the pressure drop over the column; this may be a limiting factor determining the maximum possible flow rate, the maximum column length, and the possibility of scaling up to columns of large diameters (i.e., the pressure specification is often reduced for columns of large diameter). However, in this case the column length can be traded off for an increase in column diameter to keep the bed volume high (also see Strategic Planning, Selecting Matrix and Column for Desalting).

To maximize the sample volume that can be applied to a column

A gel matrix should be selected from which the protein is excluded (see Strategic Planning) and in which the contaminants are eluted close to the total liquid volume (see Basic Protocol 1). It should also have a small matrix volume.

The maximum sample volume that can be applied to a column is directly related to the pore volume of the packed bed (V_i). The pore volume is proportional to the geometric column volume. As a rule of thumb, the maximum sample volume that may be applied for desalting is 25% to 40% of the bed volume for the materials listed in Table 8.3.1. The lower figure applies to small columns (e.g., <10-ml bed volume) and the higher figure to large columns (e.g., 100-ml bed volume). After the bed length

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is determined on basis of the acceptable pressure drop (which is proportional to the bed length), the sample capacity is maximized by increasing the diameter of the column. This is done because the applicable volume is directly proportional to the cross-sectional area of the column. Limitations in applicable column length may be eliminated by using columns in series ("stacked columns").

The maximum volume that theoretically can be applied for desalting is equal to the separating volume of the system, which is calculated by subtracting the void volume from the total volume (see Basic Protocol 1 for steps to determine these quantities). To account for extra zone-broadening effects, it is generally advisable to apply not more than 80% of the separating volume or even less—e.g., 50%—for small columns in which eddy dispersion (see discussion below) will contribute proportionally more to the zone broadening.

To optimize the yield of desalting

Sampling of the protein fraction must be adjusted according to the zone broadening of the sample (i.e., the width of the protein zone). For a well-packed column with a plate height (see Basic Protocol 2) roughly equal to two particle diameters, this parameter may be estimated as the sample volume plus the dispersion of the zone that occurs through different pathways in the packed bed—i.e., eddy dispersion. The peak width (w_b) is described by Equation 8.3.1:

$$w_b = 4(V_t)\left(\frac{2d_p}{L}\right)^{\frac{1}{2}}$$

Equation 8.3.1

where V_t is the total liquid volume of the column, d_p is the particle size of the matrix, and L is the length of the packed bed. Thus, for a 5-ml NAP-5 column (0.9 × 2.8–cm bed dimensions) packed with Sephadex G-25 medium-particle matrix (particle size 175 µm), eddy dispersion adds ~0.6 ml to the sample volume. This is roughly equal to the maximum sample volume (i.e., the separation volume; see Basic Protocol 1) and thus the eluted fraction is twice the sample volume. However, for a much larger 4 × 85–cm column packed with Sephadex G-25 coarse-particle matrix, which has an estimated particle size of 300 µm, the eddy dispersion adds ~100 ml to the originally applied sample volume of 400 ml, giving a protein zone of 500 ml (see Fig. 8.3.3). If G-25 medium-particle matrix were used, the smaller particle size would in this case theoretically decrease the protein zone from 500 to 475 ml, at the expense of a pressure drop over the column three times higher than with the coarser medium.

To minimize the time of the separation

Use the highest possible flow rate for the desalting step. High flow rates are suitable for desalting because eddy dispersion effects are rather insensitive to flow rate. The limiting factors on the flow rate are the rigidity of the matrix and the pressure limitations of the system used. Because media with small pore volume are generally more rigid than those with large pore volume, the disadvantage of a smaller separating volume may be offset by increasing the applicable flow rate to keep productivity high when working with the former type of medium. Incomplete removal of fine particles from the medium prior to packing may reduce the flow rate of the column after use as a result of partial blockage of the outlet filter. Operating the column with the flow direction reversed may help to increase the applicable flow rate in such situations.

The total process time for repetitive runs to desalt large volumes may be further minimized by utilizing the dead time of the void fraction. To do this, samples are added, not after total liquid volume + sample volume + eddy dispersion volume, but after subtracting the void volume from the total process volume (i.e., the next sample is added before the salt peak of the previous sample has emerged). The saving in time may be in the range of 20% to 30%, but this practice can only be recommended for well-understood processes.

Optimization and scaleup of protein fractionation

There are two basic ways to optimize the resolution of a separation. One is to increase the peak-to-peak distance and the other is to decrease the peak widths. The resolution for gel filtration is described by Equation 8.3.2:

$$R_s = \frac{1}{4}\log_{10}\left(\frac{M_{r1}}{M_{r2}}\right)b\left[\frac{1}{\left(\frac{V_o}{V_i} + K_D\right)}\right]\left(\frac{L}{H}\right)^{\frac{1}{2}}$$

Equation 8.3.2

where M_{r1} and M_{r2} are the molecular masses of the two sample components being separated, b is the slope of the selectivity curve

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($-\partial K_D/\partial \log M_r$), V_o is the void volume of the column, V_i is the pore volume of the bed, L the bed height, and H the plate height of the column (i.e., the term $L/H = N$, the plate number). The impact of these parameters will be described below.

To increase the peak-to-peak distance

1. A matrix of high selectivity should be chosen. The most important factor for optimizing protein fractionation is selectivity, which is the inherent ability of the matrix to resolve proteins of similar size. This factor is a function of the pore-size distribution of the matrix. Selectivity is often illustrated by a plot of the elution volumes of a series of reference substances versus the logarithm of the molecular size or molecular mass of those substances. A function of the elution volume (e.g., the distribution coefficient; see Basic Protocol 3) may be substituted for the elution volume in constructing a selectivity curve. The steeper the selectivity curve (i.e., the greater the absolute value of the slope b, defined above), the better the resolvability of proteins differing in size. The selectivity of different gels can be judged from the fractionation range stated by the manufacturer. Gels with a small fractionation range (in terms of $\log M_r$) will generally have higher selectivity than gels with a large fractionation range.

2. A gel of high pore volume should be selected. The pore volume of a matrix is often related to the particle size. Smaller particles need to resist a higher friction force from the high flow rates that are used with matrices of smaller bead size; therefore the matrix volume is generally larger and pore volume smaller when using these matrices. A traditional soft-gel matrix may have up to 98% pore volume, whereas rigid matrices such as silica may have as low a pore volume as 52% (of the bead volume).

3. A column of sufficient length (L) should be selected. The resolution between peaks is proportional to the square root of the column length. The column length chosen for protein fractionation is usually optimized with respect to the particle size of the matrix—i.e., a 100-cm column is often chosen where the particle size is 100 µm, a 60-cm column where the bead size is 30 µm, and a 30-cm column where the bead size is 10 µm.

To decrease peak width

1. A material that yields a small void volume (V_o) should be selected. Reducing the void volume will decrease the dispersion of the protein band (as will minimization of all extra column dead volumes). Irregularly shaped or rigid particles generally yield beds with larger void volumes than spherical, nonrigid beads.

2. A material of small particle size should be used, as this will affect the plate height (H; also see Basic Protocol 3). Zone broadening of proteins within a column is directly proportional to the square of the particle size. Therefore, smaller particles will yield smaller peak widths unless very large sample volumes are applied or large system effects are causing dispersion of zones.

3. The separation should be run at optimum flow rate, as this will affect the plate height (see Basic Protocol 3). Zone broadening is caused by dispersion within the void volume of the chromatographic bed, or by nonequilibrium effects that occur in the separation process itself when the mobile-phase velocity in the extra-particle space is too high as compared with the diffusivities of the proteins and the diffusion distances in the matrix. Flow rates that will allow minimum zone broadening and complete equilibrium will in most cases be impractically low. Table 8.3.7 lists practical flow rates for different solute molecular weights and matrix particle sizes, which represent compromises with regard to these parameters.

Dissolving the sample

Care must be taken to assure that the sample is completely dissolved in the GF buffer. Conditions must be chosen so that spontaneously formed aggregates are dissolved and aggregates that are to be removed are not dissolved merely to reaggregate in the fraction tube. Because gel filtration permits rather free choice of pH, ionic strength, and additives, it is generally not difficult to find suitable conditions.

Solute-matrix interactions

Solute-matrix interactions are generally to be avoided. Even though mixed-mode separations (i.e., separations on the basis of both molecular size and the solute-matrix interaction) have sometimes given good resolution, one must be concerned about the reproducibility of such mechanisms. Solute-matrix interactions may be specific or nonspecific, and the chemical basis may be an ionic or hydrophobic interaction. In the case of specific solute-matrix interactions, the only thing to do is to change the matrix to one based on a different chemistry. Ionic interaction can be suppressed by addition of an electrolyte; hydrophobic interaction can

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be reduced by adding an organic modifier (e.g., methanol, acetonitrile, or isopropanol) or by reducing the ionic strength of the buffer. Of course, avoidance of solute-matrix interactions is a prerequisite for estimating molecular size using a calibrated column (see Basic Protocol 3 and Support Protocol). Solute-matrix interactions can be uncovered by varying the composition of the mobile phase or temperature and noting the effect. Because ideal gel filtration is driven by entropy, temperature should not affect the elution volume of proteins (unless the temperature change affects protein size or the pore size).

Column maintenance

Column life is drastically reduced by introduction of air into the column, deposition of material on the bed surface, and use of flow rates that exceed the maximum recommended flow rate. To protect valuable columns from air it is advisable to use an air trap, to degas the buffer, or to use a deaerator. Deposition of material is minimized by filtering both sample and buffer through a 0.22-μm filter prior to application. Exceeding the maximum flow rate or pressure (e.g., by failing to compensate for viscosity effects at low temperatures) will result in the bed becoming compressed. This may be avoided by using a flow or pressure sensor to shut down the pump if the limit is exceeded. To maintain the calibration of a column for molecular size determinations (see Basic Protocol 3 and Support Protocol), the flow through the column should never be turned off completely, but may be reduced (e.g., to one-tenth) and the effluent recycled to the buffer reservoir through a filter. The buffer should be treated with preservatives and must be discarded when the column is put back in service. According to the author's experience, the lifetime of a column may be expected to exceed several years and calibration may remain valid for up to 1 year if the appropriate precautions are taken.

A parameter that reflects the possibility of bed compression is the void fraction—i.e., the void volume divided by the bed volume. This parameter is 0.26 for beds made up of tetrahedral, closely packed spheres and 0.40 for beds made up of randomly packed spheres. Experimentally determined void fractions vary from 0.30 to 0.47 because of the surface and packing properties of the matrix and the particle shape. Void fractions <0.30 may indicate compression of beads.

Flow rate

Flow rate must be carefully controlled, especially if the column is used for characterization of molecular size as in Basic Protocol 3. The actual flow rate of a pump seldom agrees with the nominal flow rate, so it is essential that the flow rate be checked regularly.

Troubleshooting

Gel filtration is a comparatively uncomplicated separation technique and troubleshooting is straightforward. Provided that the critical aspects of the technique (see Critical Parameters) are kept under control, gel filtration can be expected to be relatively trouble-free. Problems that may arise most frequently are compression of the bed and consequent decrease in flow rate, air bubbles in the column, tailing or leading peaks, and peaks that elute in the "wrong place."

Bed compression and decreased flow rate

Compression of the bed most likely results from the maximum flow rate being exceeded during packing (see Critical Parameters). It may also arise from excessive packing of the column, which creates an inhomogeneously packed bed with a denser layer in the lower part of the column that will eventually collapse even at a moderate flow rate. A column found to have a compressed bed must be repacked.

Compression may also result from partial clogging of the inlet filter and the upper part of the bed. To save the column in such a case, the adaptor should be removed, the filter cleaned, contaminated upper matrix removed, the upper part of the bed swirled, and the bed restabilized (see Basic Protocol 1, step 11). If the column still does not perform as expected, it must be repacked.

Decrease in the flow rate often accompanies compression of the bed. If the gel contains fines, these may block parts of the outlet net, causing a decrease in flow rate. Reversing the direction of flow may restore normal conditions. If this is not successful, the column should be repacked after exhaustive decantation of fines (see Basic Protocol 1, steps 3a and 2b).

Air bubbles in column

Small air bubbles in the column may be due to failure to deaerate the buffer or to the introduction of bubbles during sample injection. Moving the column from a cold room directly to a warmer laboratory will also release air bubbles in the column and should be avoided.

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An attempt may be made to remove the bubbles by pumping deaerated buffer through the column in the reverse direction. Heating the buffer slightly is sometimes successful, as the temperatures of the buffer and gel will equilibrate, allowing the warmer buffer to dissolve some air. This may have to be done at least overnight and perhaps for several days to be fully successful. A large air pocket situated right under the adaptor that has not been taken up by the buffer may be removed using the procedures for readjusting the adaptor to the bed surface (see Basic Protocol 1, step 11).

Tailing and leading peaks

Tailing peaks indicate a large dead volume somewhere in the system, solute-matrix interactions, or a bed that is packed too loosely. To check the system for extra dead volumes, another column or a dummy column of small volume should be packed and a sample run to see if the problem reproduces itself. If it does not, the problem can be attributed to the column, and a sample of a different substance should then be run to ensure that the effect is not caused by solute-matrix interactions. If the tailing problem is still noted, the defect probably lies in the column packing, and an attempt should be made to stabilize the bed at a higher flow rate (see Basic Protocol 1, step 11). If this does not help, the column should be repacked at a higher flow rate. Before doing this, however, it should first be ascertained that the tailing is really seen with the sample to be run under the actual conditions under which it is to be run. Slight tailing of a small solute may be negligible when compared to a large diffusion-regulated zone broadening of a protein. Tailing may also be caused by partial separation of a small amount of a solute of slightly smaller size or slightly different surface properties.

Leading (fronting) peaks indicate solute-matrix interactions or cracks in the bed. Solute-matrix interactions (e.g., ionic-exclusion effects) are detected by running a different substance and/or using a different buffer. If the problem is related to the column packing (i.e., cracks in the bed), the column must be repacked at a lower flow rate. As with tailing peaks, however, the actual sample should first be run under the actual conditions to be used to determine whether the effect of fronting on the separation is serious. Fronting may also be caused by partial separation of a small amount of slightly larger contaminant.

Peaks that elute in the wrong place

Peaks that elute prior to the void volume indicate ionic-exclusion effects. To suppress these effects, the ionic strength of the GF buffer should be increased. Solutes that elute after the total liquid volume indicate adsorptive effects. These may be masked by adding an organic modifier or by increasing or decreasing the ionic strength of the buffer (see Critical Parameters for discussion of solute-matrix effects).

Anticipated Results

Results from gel filtration are in general very predictable. Guidelines for optimization and scaleup are presented in Hagel et al. (1989) and Hagel and Janson (1992).

Time Considerations

Gel filtration using media of small particle size may generally be carried out in half an hour. Gel filtration using traditional larger-particle matrices are typically run for 5 hr. Desalting is very fast: e.g., 1.4 ml of sample may be desalted in 30 sec.

Preparation (i.e., swelling) of the gel will take 3 hr to overnight, depending on the procedure chosen. Packing of the column takes 1 to 4 hr, and the subsequent stabilization of the packed bed takes 1 hr. Thus, a column is typically prepared in a day, and testing of the packing quality is carried out overnight.

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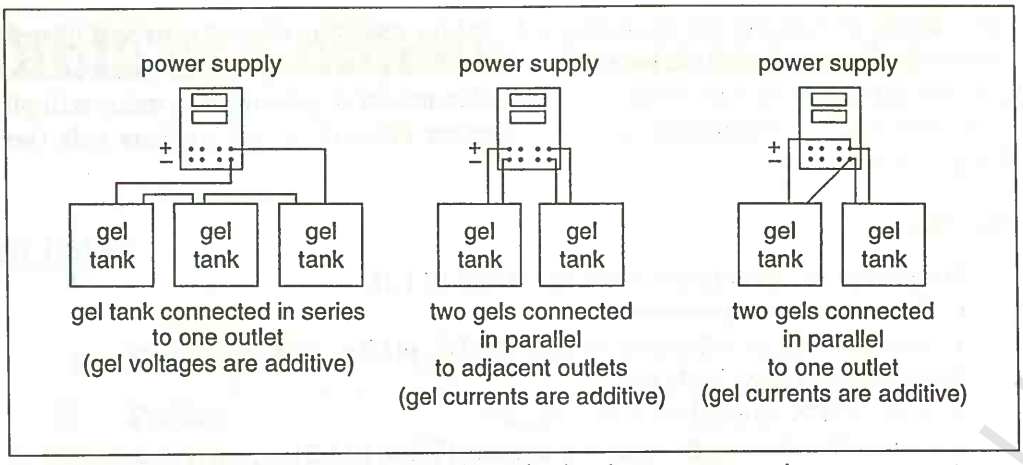

Figure 10.1.1 Series and parallel connections of gel tanks to power supply.

increase the current for each additional gel that is connected to the power supply. Two identical gels require double the current to achieve the same starting voltages and electrophoresis separation times.

Multiple gel apparatuses can also be connected to one pair of outlets on a power supply. This is useful with older power supplies that have a limited number of outlets. When connecting several gel units to one outlet, make certain the connections between the units are shielded and protected from moisture. The gels can be connected in parallel or in series (Fig. 10.1.1). In the case of two or more gels running off the same outlet in series, the current is the same for every gel. If 10 mA is displayed by the power supply meter, for example, each gel in series will experience 10 mA. The voltage, however, is additive for each gel. If one gel at a constant 10 mA produces 100 V, then two identical gels in series will produce 200 V (100 V each) and so on. Thus, the voltage can limit the number of units connected in series on low-voltage power supplies.

Gel thickness affects the above relationships. A 1.5-mm gel can be thought of as consisting of two 0.75-mm-thick gels run in parallel. Because currents are additive in parallel circuits, a 0.75-mm gel will require half the current of the 1.5-mm gel to achieve the same starting voltage and separation time. If a gel thickness is doubled, then the current must also be doubled. There are limits to the amount of current that can be applied. Thicker gels require more current, generating more heat that must be dissipated. Unless temperature control is available in the gel unit, a thick gel should be run more slowly than a thin gel.

NOTE: Milli-Q-purified water or equivalent should be used throughout the protocols.

DENATURING (SDS) DISCONTINUOUS GEL ELECTROPHORESIS: LAEMMLI GEL METHOD

BASIC PROTOCOL 1

One-dimensional gel electrophoresis under denaturing conditions (i.e., in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel (sometimes called resolving or running gel) topped by a stacking gel and secured in an electrophoresis apparatus. After sample proteins are solubilized by boiling in the presence of SDS, an aliquot of the protein solution is applied to a gel lane, and the individual proteins are separated electrophoretically. 2-Mercaptoethanol (2-ME) or dithiothreitol (DTT) is added during solubilization to reduce disulfide bonds.

This protocol is designed for a vertical slab gel with a maximum size of 0.75 mm × 14 cm × 14 cm. For thicker gels, or minigels (see Basic Protocol 2 and Support Protocol 3),

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the volumes of stacking and separating gels and the operating current must be adjusted. Support protocols describe the preparation of ultrathin gels (see Alternate Protocol 4) and gradient gels (see Alternate Protocol 5), as well as the use of gel casters to make multiple gels, both single-concentration gels (see Support Protocol 1) and gradient gels (see Support Protocol 2).

Materials

Separating and stacking gel solutions (Table 10.1.1)
H_2O-saturated isobutyl alcohol
1× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 10.1.1)
Protein sample to be analyzed
2× and 1× SDS sample buffer (see recipe)
Protein molecular-weight-standards mixture (Table 10.1.2)
6× SDS sample buffer (see recipe; optional)
1× SDS electrophoresis buffer (see recipe)

Electrophoresis apparatus: Protean II 16-cm cell (Bio-Rad) or SE 600/400 16-cm unit (Hoefer Pharmacia) with clamps, glass plates, casting stand, and buffer chambers
0.75-mm spacers
0.45-µm filters (used in stock solution preparation)
25-ml Erlenmeyer side-arm flask
Vacuum pump with cold trap
0.75-mm Teflon comb with 1, 3, 5, 10, 15, or 20 teeth
25- or 100-µl syringe with flat-tipped needle
Constant-current power supply (see introduction)

Pour the separating gel

1. Assemble the glass-plate sandwich of the electrophoresis apparatus according to manufacturer's instructions using two clean glass plates and two 0.75-mm spacers.

   If needed, clean the glass plates in liquid Alconox or RBS-35 (Pierce). These aqueous-based solutions are compatible with silver and Coomassie blue staining procedures.

2. Lock the sandwich to the casting stand.

3. Prepare the separating gel solution as directed in Table 10.1.1, degassing using a rubber-stoppered 25-ml Erlenmeyer side-arm flask connected with vacuum tubing to a vacuum pump with a cold trap. After adding the specified amount of 10% ammonium persulfate and TEMED to the degassed solution, stir gently to mix.

   Table 10.1.1 was prepared as a convenient summary to aid in the preparation of separating and stacking gels. The stacking gel is the same regardless of the separating gel used.

   The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. Generally, use 5% gels for SDS-denatured proteins of 60 to 200 kDa, 10% gels for SDS-denatured proteins of 16 to 70 kDa, and 15% gels for SDS-denatured proteins of 12 to 45 kDa (Table 10.1.1).

4. Using a Pasteur pipet, apply separating gel solution to the sandwich along an edge of one of the spacers until the height of the solution between the glass plates is ~11 cm.

   Use the solution immediately; otherwise it will polymerize in the flask.

   Sample volumes <10 µl do not require a stacking gel. In this case, cast the resolving gel as you normally would, but extend the resolving gel into the comb to form the well. The proteins are then separated under the same conditions as used when a stacking gel is present. Although this protocol works well with single-concentration gels, a gradient gel is recommended for maximum resolution (see Alternate Protocol 5).

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Sample Preparation for MALDI Mass Analysis of Peptides and Proteins

The discovery that polar biological substances may be ejected from solid and liquid matrices as protonated or deprotonated ions by deposition of laser pulses initiated the development of methodology and instrumentation for characterization of biomacromolecules at unprecedented sensitivities. This is generally known by the acronym, MALDI-MS, for matrix-assisted laser desorption/ionization mass spectrometry (also see *UNITS 16.1 & 16.2*). The physicochemical properties of matrices that work well analytically are diverse; some matrices are suited to UV/visual laser wavelengths and others to the infrared. Although originally thought to be "soft" in terms of minimal deposition of internal energy (in the case of UV/visual laser frequencies), most modulate the vibronic activation of the analyte, giving rise to a fraction of metastable ions that are formed by unimolecular dissociation of the $[M + H]^{1+}$ or $[M - H]^{1-}$. The recorded mass spectra of these metastable ions, known as post-source decay (PSD) mass spectra, are similar to those of low-energy collision-induced dissociation (CID) fragment ions. The preponderance of ions formed are singly charged. Thus, they are well suited to high-energy collisional activation using appropriate tandem experiments to give high-energy CID-like mass spectra.

Sample preparation may be the most crucial step in mass spectrometric analysis of peptides and proteins by MALDI (also see sample preparation guidelines in *UNIT 16.2*). The free dipolar ionic peptides or proteins must be incorporated into a crystalline solid solution that is comprised of excess solvent (the matrix), which modulates desorption/ionization by preferentially absorbing the laser energy at the irradiation wavelength and laser pulse intensity being employed. Usually the cocrystalline sample and matrix are prepared by mixing solutions of each component and permitting the mixture to crystallize through evaporation of the solvents. Two methods employing this approach are presented in this unit: the dried drop method (see Basic Protocol 1) and the rapid evaporation method (see Basic Protocol 2); also see Background Information and Table 16.3.1). This process can be disturbed by the presence of contaminants such as detergents and salts (see Table 16.2.1). Matrices that are sufficiently water insoluble will permit a degree of on-target washing without dissolving and liberating the analyte. Such matrices are useful for removing some contaminants, and include α-cyano-4-hydroxy-*trans*-cinnamic acid (HCCA) and sinapinic acid. Alternatively, the water-soluble 2,5-dihydroxybenzoic acid (DHB) may be used, since this matrix excludes some impurities during crystallization.

DRIED DROPLET METHOD

BASIC PROTOCOL 1

Materials

100% methanol
Peptide or protein sample of interest: ~1 pmol/μl in 0.1% (v/v) trifluoroacetic acid (TFA)
Peptide or protein matrix solution (see recipes)
Peptide or protein standard mixture (see recipes)
Matrix-assisted laser desorption/ionization (MALDI) mass spectrometer

1. Clean the sample target with the appropriate solvents. Wipe with a Kimwipe wetted with water followed by one wetted with 100% methanol, and repeat for several cycles.

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This cleaning procedure is usually sufficient. Alternatively, wipe with 0.1% (v/v) TFA and/or sonicate the target in 100% methanol or 0.1% TFA. Detergents are not recommended as they are hard to wash away and tend to give background peaks.

2. Pipet 0.5 to 1 µl peptide or protein sample onto the target, add 0.5 to 1 µl peptide or protein matrix solution, and mix by pipetting up and down. Allow to air dry.

   Samples may also be prepared by mixing the sample and matrix solution in a microcentrifuge tube, from which 0.5 to 1 µl is applied to the target.

3. For external calibration, apply 0.5 to 1.0 µl of an appropriate peptide or protein standard mixture and 0.5 to 1.0 µl peptide or protein matrix solution (as in step 2) to the target in the vicinity of the sample.

   For highest mass accuracy, the standard solution may be mixed with an aliquot of the sample for internal calibration. However, do not add an excess of internal standard, as this may result in ion suppression of the sample. The optimal ratio of sample/standard may have to be empirically determined, although starting with equimolar amounts is recommended. In the case of peptide analysis, internal calibration may be performed on matrix ions.

4. Insert sample target into the MALDI mass spectrometer and mass analyze as recommended by the manufacturer.

   The sample target with applied matrix/sample preparations may be stored in the dark at ambient temperature for days without significant loss of signal. For longer storage, the target should be kept at –20°C in a sealed container.

   Irradiate the sample just above the threshold for appearance of the ions. Generally, the ion signals (mass-to-charge ratio, m/z) of peptides correspond to $[M + H]^{1+}$. Larger peptides and proteins yield doubly protonated ions $[M + 2H]^{2+}$ that appear at half the peptide or protein mass since m/z is being measured.

   Due to microheterogeneity of the matrix crystals, spectra should be accumulated from several different crystals of the matrix/sample preparation to obtain a representative sum spectrum. Under certain circumstances, the relative peak abundances may give a semiquantitative indication of the relative concentrations of peptides in a mixture.

BASIC PROTOCOL 2

RAPID EVAPORATION METHOD

Materials

100% methanol
Peptide matrix/nitrocellulose solution (see recipe)
Peptide sample of interest: ~1 pmol/µl in 0.1% (v/v) trifluoroacetic acid (TFA)
10% (v/v) formic acid, 0° to 4°C
Peptide standard mixture (see recipe)
Matrix-assisted laser desorption/ionization (MALDI) mass spectrometer

1. Clean the sample target with the appropriate solvents (see Basic Protocol 1, step 1).

2. Pipet ~0.5 µl peptide matrix/nitrocellulose solution on the sample target. Pipet quickly because of the high volatility of the solvent.

   The matrix/nitrocellulose solution will spread out rapidly, allowing fast evaporation of the solvent and the formation of a uniform white layer.

3. Apply ~0.5 µl peptide sample onto the matrix surface on the sample target. Allow the solvent to evaporate at ambient temperature.

   The sample solution should not completely dissolve the matrix layer. Therefore, the concentration of organic solvent in the sample should be kept <30% and the pH should be acidic. For samples with higher organic solvent concentrations or high pH, apply 0.5 µl

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water or 0.5 µl of 10% (v/v) formic acid, respectively, onto the matrix surface and then apply the sample.

4. Rinse the sample with ~10 µl cold 10% formic acid. Leave the solution on the sample for 10 sec and then remove using pressurized air, vacuum, or careful pipetting. Repeat the rinse step with water.

   This step is optional and can be omitted for samples that are free of salts (e.g., reversed-phase HPLC fractions). These rinse steps are strongly recommended for unseparated peptide digest mixtures.

5. Apply peptide standard mixture (for details on internal versus external standards, see Basic Protocol 1, step 3).

6. Insert sample target into the mass analyzer and analyze (see Basic Protocol 1, step 4).

   For best ion intensities, matrix/sample preparations made using the rapid evaporation method should be analyzed immediately.

PURIFICATION/RECRYSTALLIZATION OF HCCA

SUPPORT PROTOCOL

α-Cyano-4-hydroxy-*trans*-cinnamic acid (HCCA) matrix that has been purified often yields better sensitivities than matrix used as supplied by the manufacturer. The HCCA cleanup protocol described below was developed by B.O. Keller (Department of Chemistry, University of Alberta).

Materials

α-Cyano-4-hydroxy-*trans*-cinnamic acid (HCCA; Sigma or Aldrich)
95% (v/v) ethanol: 50°C, room temperature, and 4°C

Class C and E Whatman glass filters
50°C water bath

1. Suspend 7 to 8 g HCCA in 100 ml of 95% ethanol at room temperature. Dissolve all brownish crumbs, so that only bright yellow crystals remain undissolved.

2. Filter suspension through a class C Whatman glass filter under a vacuum. Wash the remaining crystals with 50 ml of 95% ethanol.

   The yield of undissolved HCCA is ~50%.

3. Dry the crystals and then pick out and discard any remaining black or brown crumbs in the dried residue.

4. Warm 50 ml of 95% ethanol in an Erlenmeyer flask or beaker in a 50°C water bath. To obtain a saturated solution, add dried HCCA crystals to the ethanol while swirling the flask, and continue until no more HCCA dissolves and yellow crystals remain undissolved at the bottom.

   The temperature should not exceed 50°C, as this may cause the HCCA to decompose.

5. Filter the hot (50°C) HCCA solution using a class C Whatman glass filter. Use a very clean glass flask to collect the filtrate. Cool to room temperature, cover flask, and leave at –20°C overnight.

   The next day, a precipitate of tiny crystals should be observed. If no precipitate is observed, the HCCA solution may be too dilute. Add more HCCA from step 3 to the solution in a 50°C water bath and filter again (step 4).

6. Separate the precipitate from the HCCA solution by filtration using a class E Whatman glass filter. Wash the precipitate moderately with cold (~4°C) 95% ethanol.

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7. Cover the purified HCCA precipitate in the glass filter with foil. Dry 3 to 4 hr, preferably in a laminar-flow hood, and transfer to glass vials. Store below 0°C (stable for years, if repeated freezing/thawing is avoided).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Peptide matrix/nitrocellulose solution

Dissolve 10 mg nitrocellulose and 40 mg/ml α-cyano-4-hydroxy-*trans*-cinnamic acid (HCCA; Sigma or Aldrich) in 1 ml acetone. If necessary, vortex 1 to 2 min to dissolve. Add 1 ml isopropanol (final 20 mg/ml HCCA and 5 mg/ml nitrocellulose in acetone/isopropanol). Prepare fresh before each use.

Peptide matrix solution

Prepare 20 mg/ml α-cyano-4-hydroxy-*trans*-cinnamic acid (HCCA; Sigma or Aldrich) either in 0.1% (v/v) trifluoroacetic acid (TFA)/50% (v/v) acetonitrile *or* in 99:1 (v/v) acetone/water (also see Support Protocol 1). Alternatively, use 20 mg/ml 2,5-dihydroxybenzoic acid (DHB) in 0.1% (v/v) TFA/30% (v/v) acetonitrile. Divide into aliquots and store at –20°C (stable for months to years).

With DHB, the percentage of acetonitrile may be varied depending on the hydrophobicity of the peptide(s) analyzed. The matrix additive 2-methoxy-5-benzoic acid may be added at 1 mg/ml to the DHB matrix solution for better detection of larger peptides (>3000 Da).

Peptide standard mixture

Prepare a mixture of peptides with known masses (e.g., bradykinin, mellitin, and ACTH) at ~1 pmol/µl in 0.1% (v/v) TFA. Divide into aliquots and store at –20° or –80°C (stable for years).

Protein matrix solution

Prepare 20 mg/ml sinapinic acid in 0.1% (v/v) trifluoroacetic acid (TFA)/50% (v/v) acetonitrile *or* in 99:1 (v/v) acetone/water. Divide into aliquots and store up at –20°C (stable for years).

Protein standard mixture

Prepare a mixture of insulin, myoglobulin, and BSA at ~1 pmol/µl (v/v) in 0.1% TFA. Divide into aliquots and store at –20°C (stable for years).

COMMENTARY

Background Information

Different on-target sample preparation methods have been developed for MALDI mass spectrometry. The most frequently used is the dried droplet method, in which the matrix is simply added to the sample on the sample stage (see Basic Protocol 1 above; see *UNIT 11.6*, Basic Protocol 3). Alternatively, a thin layer of matrix material dissolved in a rapidly evaporating solvent may be prepared on the sample target holder, onto which a solution of the analyte sample is subsequently deposited to avoid complete dissolution of the preprepared matrix film. The latter method has provided improved sensitivity and uniform desorption properties (Vorm et al., 1994). Moreover, the addition of nitrocellulose to the matrix solution may be employed to achieve better peptide binding to the target, allowing more extensive rinsing of the sample (see Basic Protocol 2; also Alternate Protocol of *UNIT 11.6*; Shevchenko et al., 1996).

Critical Parameters and Troubleshooting

The method of sample preparation as well as the matrix solvent composition can significantly affect results (Table 16.3.1) due to differential mass discrimination and ion suppression effects (Cohen and Chait, 1996). This is especially true when complex peptide mixtures

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are analyzed, such as unseparated protein digests. For example, in tryptic digest mixtures, the C-terminal arginine-containing components are preferentially detected. Therefore, to obtain better detection of most of the components in such a mixture, spectra should be acquired using different matrix solution conditions and different matrices.

Contaminants such as salts and detergents should be <50 mM. When crystallization is impaired due to the presence of contaminants, the matrix/sample preparation should be rinsed 1 to 3 times with ice-cold 0.1% (v/v) TFA or 10% (v/v) formic acid, and the solvent should be removed after a few seconds by pipetting or with blown pressurized air. However, hydrophilic peptides may be lost in this washing procedure, and it is not suitable for DHB matrix solutions. Alternatively, the sample can be diluted and reapplied to the target. Volatile buffers may be removed by partial drying with vacuum centrifugation. Complete lyophilization is not

Table 16.3.1 Effects of Matrix Solution Composition, pH, Presence of Detergent, and Crystallization Rate on MALDI-MS of a Complex Mixture of Peptides[a]

		Relative peptide ion intensity		
	pH	0.8-2 kDa	2-6 kDa	6-11 kDa
Composition of HCCA solution				
0.1 N HCl/ACN (2:1)	1.1	+	+++	++
1% TFA/ACN (2:1)	1.1	+	+++	++
Formic acid/water/ACN (1:3:2)	1.1	+	++	+++
Formic acid/water/IPA (1:3:2)	1.3	+	++	+++
0.5% TFA/ACN (2:1)	1.4	+	+++	++
Formic acid/water/methanol (1:3:2)	1.6	+	++	+++
0.01 N HCl/ACN (2:1)	1.9	++	+++	++
Formic acid/water/IPA (0.2:3:2)	2.0	++	+++	++
0.1% TFA/ACN (2:1)	2.0	++	+++	++
0.1% TFA/methanol (2:1)	2.0	++	+++	++
0.1% TFA/IPA (2:1)	2.1	++	+++	++
2 M acetic acid/ACN (2:1)	2.3	++	+++	++
Water/ACN (1:1)	2.4	+++	++	+
Water/ACN (2:1)	2.5	+++	++	+
Water/IPA (2:1)	2.7	+++	++	+
Water/ethanol (2:1)	2.8	+++	++	+
Water/methanol (2:1)	2.9	+++	++	+
N-Octyl-β-D-glucoside				
Water/ACN (2:1) alone		+++	++	+
20 mM in water/ACN (2:1)		+++	+++	+++
Rate of crystallization				
Formic acid/water/IPA (1:3:2)				
Slow		+	+++	ND
Dried droplet		+	+++	ND
Rapid		+++	+	ND
Water/ACN (2:1)				
Slow		+	+++	ND
Dried droplet		++	++	ND
Rapid		+++	+	ND

[a]Peptide mixture is a partial V8 protease digest of the 11 kDa protein Max (for details see Cohen and Chait, 1996). The dried droplet method (see Basic Protocol 1) was used for the analysis of the effect of matrix composition/pH and *N*-octyl-β-D-glucoside on MALDI-MS performance. For each matrix condition, the three mass ranges are assigned a response level: +++ designates the mass range with the highest relative ion intensities; ++ indicates moderate ion intensities; + indicates weak or no peptide ions. Abbreviations: ACN, acetonitrile; IPA, isopropyl alcohol; ND; not determined; TFA, trifluoroacetic acid.

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recommended because of severe sample losses. A partially lyophilized sample can be diluted with 0.1% to 5% (v/v) TFA in 20% (v/v) acetonitrile to reduce adsorptive losses and to facilitate MALDI mass analysis.

Anticipated Results

Using MALDI-MS, peptides generally yield singly protonated ions, and reasonable signal-to-noise ratios can be obtained, even in the low femtomole range. When salt is present in the sample, sodium (+22.99) and potassium (+39.10) adducts may be observed in the spectrum as well.

Mass accuracy for linear time-of-flight (TOF) mode with external calibration is typically ±2 to 3 Da for peptides <3000 Da. Reflector-TOF instruments offer improved mass accuracy with external calibration (±0.5 to 1 Da), but a 5- to 10-fold loss in signal may be observed, especially for larger peptides that are more prone to metastable fragmentation. With delayed extraction in reflectron mode, the full-width, half-maximum mass resolution (M/ΔM, FWHM) can reach >10,000 Da for analytes that have mass-to-charge ratios of 1000 to ~6000 kDa, and mass accuracies in the low parts-per-million range can be achieved at low levels of gel-separated proteins (Clauser et al., 1996 and unpub. observ.). In addition, delayed extraction provides significant improvement in MALDI spectral quality, including a lower level of chemical noise arising from fragmentation in the desorption plume, reduction or elimination of matrix background, and significant reduction in the dependence of ion flight times on laser intensity (Vestal et al., 1995). Peak width and centroids are much less dependent on the absolute intensity of the ion signal. Therefore, high mass resolution and improved mass accuracy can be achieved even when the peaks are small.

Time Considerations

One of the main advantages of MALDI-MS is its speed: MALDI sample preparation only takes a few minutes and a typical MALDI mass measurement may take less than a minute, or up to 10 min when very low amounts of sample or high levels of contaminants are present.

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A comprehensive review on applications and recent developments in MS instrumentation.

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In-Gel Digestion of Proteins for MALDI-MS Fingerprint Mapping

Polyacrylamide gel electrophoresis is a widely used technique for separating proteins from biological samples. Moreover, the development of two-dimensional gel electrophoresis (*UNIT 10.4*) has provided a tool for differential protein display, which allows for the quantitative analysis of thousands of proteins simultaneously. Complex biological questions can now be approached by analyzing differences in the two-dimensional gel patterns between control and experimental states (Fig. 16.4.1; Patterson and Aebersold, 1995; Arnott et al., 1998; Qiu et al., 1998).

Although highly sensitive staining methods could visualize proteins at increasingly low levels, originally protein identification was limited by the sensitivity of Edman sequencing. However, in the last few years mass spectrometry (MS) has emerged as a more sensitive, versatile, and rapid alternative, following the advent of electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The advantages of MALDI-MS over ESI-MS include its relatively high tolerance to contamination from biological matrices, its high sensitivity, the relative ease of interpreting spectra from mixtures, and the formation of singly protonated molecular ions for tandem analysis (see *UNIT 16.6* for analysis of MALDI collision-induced dissociation, or MALDI-CID).

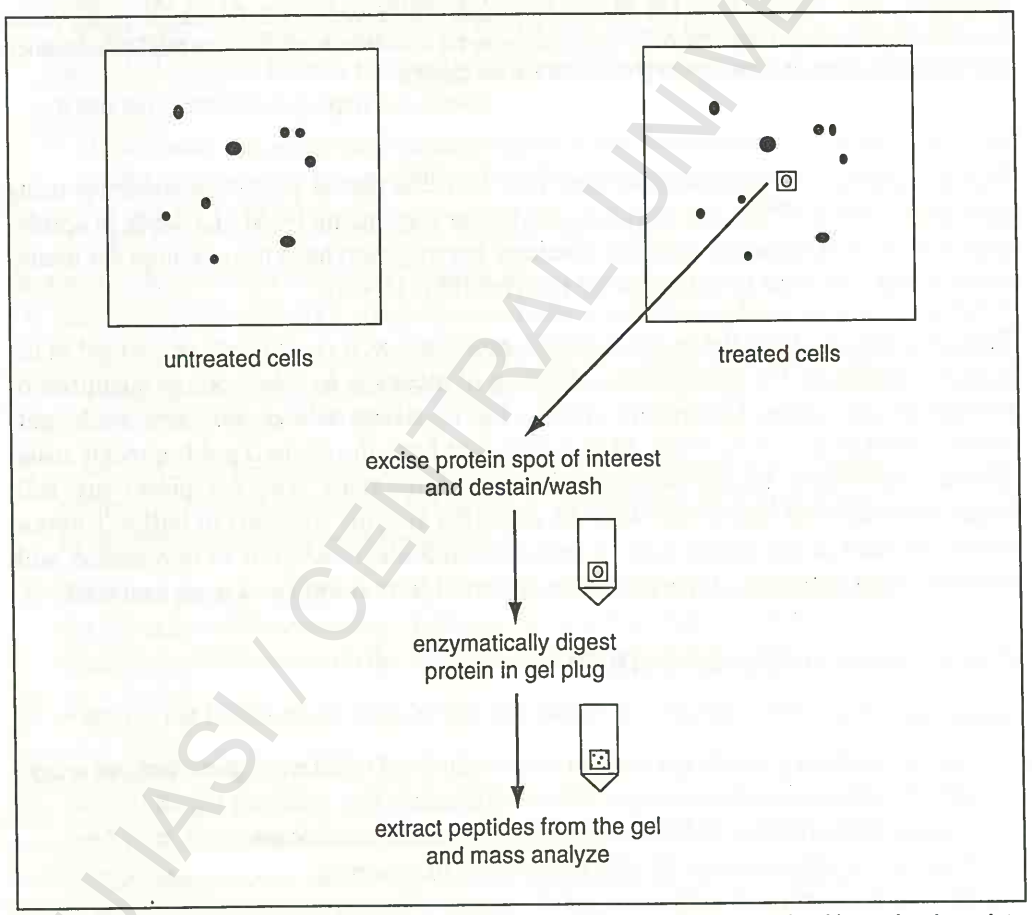

Figure 16.4.1 Protein in situ (in-gel) digestion. A spot from an SDS-PAGE gel is excised, cut into small particles (not shown), and placed in a silanized tube. The gel particles are destained, washed, dried, and rehydrated with a protease. The resulting peptides are extracted from the gel plug and subjected to mass measurement.

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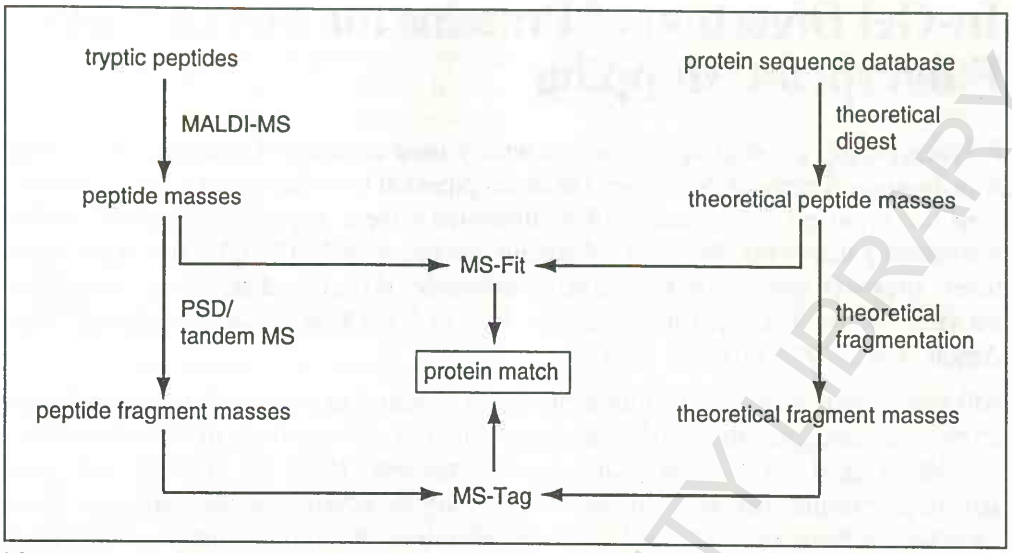

Figure 16.4.2 Peptide fingerprint mass mapping and peptide fragmentation mapping, using the database search programs MS-Fit and MS-Tag (*http://prospector.ucsf.edu*), respectively. The masses of peptides generated by enzymatic cleavage of the protein of interest (left) are matched with peptides generated by theoretical cleavage of each protein in the database (right) using the search program MS-Fit (see *UNIT 16.5* for further explanation). The masses of the post-source decay (PSD) ions or fragment ions (left) are matched with theoretical fragmentation products of each protein in the database (right) using the search program MS-Tag (see *UNIT 16.6* for details). For a conclusive protein identification, MS-Tag search results should confirm MS-Fit search results. When no reasonable matches are obtained, the protein may be unknown, in which case MS/MS sequence information is needed to design oligonucleotides for cloning.

Peptide fingerprint mass mapping (see *UNIT 16.5*) and partial peptide sequencing using post-source decay (PSD; see *UNIT 16.6*), and ladder sequencing by MALDI-MS in combination with algorithms for sequence database interrogation have the potential for identification and structural investigation of proteins (Fig. 16.4.2).

This protocol describes the in-gel digestion procedure as it is routinely performed in the authors' laboratory for peptide mass mapping of picomole to subpicomole quantities of protein derived from Coomassie- (*UNIT 10.5*) or silver-stained polyacrylamide gels (Shevchenko et al., 1996). First, SDS is removed from the excised gel fragments using several ammonium bicarbonate/acetonitrile washing steps. The gel pieces are subsequently dried and rehydrated with the digestion enzyme (trypsin) in buffer. Alternatively, the washed gel pieces may be reduced and S-alkylated prior to rehydration with enzyme. After digestion, the peptides are extracted from the gel and mass analyzed.

BASIC PROTOCOL

IN-GEL DIGESTION PROCEDURE

Materials

Stained polyacrylamide gel containing protein band of interest (*UNITS 10.1, 10.4 & 10.5*)
25 mM ammonium bicarbonate/50% (v/v) acetonitrile
10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate
55 mM iodoacetamide in 25 mM ammonium bicarbonate
25 mM ammonium bicarbonate, pH 8
0.05 to 0.1 mg/ml trypsin (sequence grade; Promega) in 25 mM ammonium bicarbonate, pH 8
5% (v/v) trifluoroacetic acid (TFA)/50% (v/v) acetonitrile
0.65-ml tubes (e.g., PGC Scientific), silanized (*APPENDIX 3E*)

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Vacuum centrifuge
56°C water bath
Sonication bath

NOTE: To avoid or reduce contamination with human keratins, one should wear gloves and preferably work in a laminar-flow hood.

Excise and wash gel fragments

1. Excise protein bands/spots of interest from a stained polyacrylamide gel. Cut each gel piece into small particles (~1 mm^2) using a scalpel, and place into a 0.65-ml siliconized tube. Also cut out and dice a gel piece from a protein-free region of the gel, for a parallel control digestion to identify trypsin autoproteolysis products.

   The amount of protein loaded on the gel must be determined empirically, as it depends on properties of the specific protein (for example, degree of hydrophobicity and size). In general, picomole levels are preferred to lower levels, although some reports claim that femtomole sensitivities are attainable with in-gel digestion and mass spectrometry.

   The small gel particle size facilitates the removal of SDS (and Coomassie) during the washes, and improves enzyme access to the gel. For Coomassie-stained proteins, one gel particle per tube is probably sufficient protein. With faint silver-stained spots, particles can be pooled, but no more than three should be pooled as this will lead to excessive levels of contaminants. For a silver-staining protocol compatible with mass spectrometry, see Shevchenko et al. (1996).

2. Add ~100 µl of 25 mM ammonium bicarbonate/50% acetonitrile (or enough to immerse the gel particles) and vortex for 10 min. Use gel-loading pipet tips to remove the solution (pale blue in the case of Coomassie staining) and discard. Repeat this wash/dehydration step up to ~3 times.

   At this point, the gel slices shrink and become white. This visual criterion should be used to determine whether or not additional washes should be performed.

3. Dry gel particles for ~30 min in a vacuum centrifuge.

Perform reduction and alkylation (optional)

4. Add enough 10 mM DTT solution to cover the gel pieces, and reduce for 1 hr at 56°C.

   Steps 4 to 7 may be included when a maximum of protein coverage is required or when digesting a band from a one-dimensional gel. Proteins separated by two-dimensional gel electrophoresis are already reduced and alkylated, so their steps can be omitted.

5. Cool to room temperature and replace the DTT solution with roughly the same volume of 55 mM iodoacetamide solution. Incubate for 45 min at room temperature in the dark with occasional vortexing.

6. Wash gel pieces (rehydrate) with ~100 µl of 25 mM ammonium bicarbonate, pH 8, for 10 min while vortexing, and dehydrate with ~100 µl of 25 mM ammonium bicarbonate/50% acetonitrile. Repeat rehydration and dehydration.

7. Remove the liquid phase and dry the gel pieces in a vacuum centrifuge.

Digest protein sample

8. Rehydrate gel particles in 1 vol of 0.05 to 0.1 mg/ml trypsin solution by vortexing for 5 min. Do not add more solution than can be absorbed by the gel particles.

   The volume needed can be estimated by calculating the total gel volume excised (e.g., 2 mm × 8 mm × 1 mm = 16 mm^3 = 16 µl).

   The enzyme-to-substrate ratio employed for in-gel digestions (>1:10) is greater than for in-solution digestions due to hindered enzyme access to the protein substrate in the gel. Moreover, the relatively low salt concentration (25 mM) is used to reduce the possibility of subsequent salt interference with ionization in the mass spectrometer.

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9. If necessary, overlay the rehydrated gel particles with a minimum amount of 25 mM ammonium bicarbonate to keep them immersed throughout digestion.

10. Incubate 12 to 16 hr at 37°C.

Recover peptides

11. Add 2 vol water, vortex for 5 min, and then sonicate for 5 min. Use a gel-loading tip to remove the peptide solution and transfer it to a silanized tube.

12. Perform two additional extractions using 2 vol of 5% TFA/50% acetonitrile.

    The silanized microcentrifuge tubes and the high TFA concentration are used to minimize adsorptive sample loss. Formic acid (5%) may be used as an alternative to TFA.

13. Concentrate recovered peptides by reducing the final volume of the extracts to ~10 µl in a vacuum centrifuge. Bring to 25 µl with 5% TFA/50% acetonitrile.

    Store the recovered peptides at –20°C until MALDI-MS is performed.

    For MALDI-MS analysis, mix 0.5 µl of the unseparated digest on the sample target with 0.5 µl of 20 mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% TFA/50% acetonitrile and let air dry (for additional details, see UNIT 16.3, Basic Protocols 1 and 2). Insert sample target into the mass spectrometer and mass analyze as recommended by the manufacturer.

14. *Optional:* Decrease the amount of volatile salts by adding a few cycles of water (100 to 200 µl) and subsequently reducing the volume in a vacuum centrifuge.

    Salt reduction may improve mass signals, particularly at subpicomole levels.

COMMENTARY

Background Information

Generally, in-gel tryptic digests of proteins yield higher recovery of peptides than proteins digested after transblotting to polyvinylidene difluoride (PVDF) membranes (*UNIT 11.2*). Trypsin is the protease of choice for peptide mass fingerprinting because of its reliability and its substrate specificity, yielding peptides with C-terminal basic residues (Arg and Lys), which facilitates ionization and subsequent mass spectrometric sequencing. However, autolysis of the protease contributes contaminating peptides that can interfere with the analysis. For trypsin, this problem is partially overcome by using modified porcine enzyme. A few labile sites are often cleaved nonetheless, resulting in peptides with monoisotopic ions of 842.51, 2211.1045, and 2284.6, which may be used for internal accurate mass calibration of the spectrum. Endoprotease Lys-C is a good alternative to trypsin, because it produces less autolysis and it is more stable under denaturing conditions. Unfortunately, endo-Lys-C yields fewer, larger fragments that may be released from the gel particles less efficiently. However, larger average lengths provide greater specificity for the search algorithm (*UNIT 16.5*).

Critical Parameters and Troubleshooting

Low concentrations of ammonium bicarbonate in the digestion buffer give the best results for subsequent mass measurement by MALDI-MS. Adsorption of the released peptides to the sample tube and other surfaces is a problem when working in the femtomole range. To prevent sample losses, digestion is performed in silanized tubes. Moreover, the presence of organic solvent (10% acetonitrile) in the digestion buffer aids in solubility without degrading enzyme activity. The use of low amounts of pure, single-component detergents (e.g., *N*-octyl-β-D-glucoside) during digestion may also improve peptide recovery.

If a higher peptide coverage of the protein is required, the amount of organic solvent and the acidity of the matrix composition can be varied (see Cohen and Chait, 1996), as can the matrix. For example, α-cyano-4-hydroxycinnamic acid is the matrix of choice for best detection limits and post-source decay analysis. However, 2,5-dihydroxybenzoic acid (DHB), or DHB with fucose, yields spectra of peptide mixtures with the least suppression characteristics. If sequence coverage is still insufficient, use a microbore liquid chromatography/ESI-MS (LC-ESI-MS) system.

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Anticipated Results

MALDI-MS analysis of unseparated tryptic digests generally yields 40% to 50% sequence coverage, although peptide recovery may be lower for hydrophobic proteins. Typically, with sufficient mass accuracy, four tryptic peptides may already be enough to identify a protein from the database using the search program MS-Tag (see *UNIT 16.6*).

Time Considerations

Generally, the in-gel digestion procedure is performed overnight. Shorter digestion times (e.g., 6 hr) are possible, but at the expense of less complete digestion.

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Searching Sequence Databases Over the Internet: Protein Identification Using MS-Fit

BASIC PROTOCOL

Peptide fingerprint mass mapping is widely used for initial identification of proteins separated by gel electrophoresis (Cottrell, 1994; Jungblut and Thiede, 1997). This method involves the enzymatic in-gel digestion of proteins to generate peptides (*UNIT 16.4*), followed by mass measurement of the cleaved peptides. The experimental mass values are then compared with theoretical values from protein databases, calculated using the cleavage specificity of the enzyme. This protocol describes the use of the search program MS-Fit, which can be accessed through ProteinProspector at *http://prospector.ucsf.edu*. By using an appropriate scoring algorithm, the closest match or matches can be identified (Cottrell, 1994).

Peptide masses can be determined using either a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) or liquid chromatography/electrospray ionization (LC-ESI) mass spectrometer. MALDI-TOF mass spectrometry has been preferred for direct analysis of unseparated digest mixtures because of its tolerance to low levels of contaminants such as buffer salts. Because of the inherently high throughput, sensitivity, and simplicity of this technique, MALDI peptide mapping is now a powerful method for identification of proteins present at greater than or equal to ~0.5-pmol on a gel (Shevchenko et al., 1996).

Input data and set search parameters

1. Input the peptide masses obtained by mass spectrometry into a spreadsheet, and then copy and paste the values into the space for masses (*m/z*) in the MS-Fit program.

   Monoisotopic peptide masses from MALDI-TOF are preferred. Additional peptide mass values obtained in parallel from multiply charged ions (m/z) using electrospray ionization mass spectrometry along with charge state (z) are submitted at the same time. Average masses may also be used for the search; however, more false positive results will be obtained due to the lower mass accuracy.

2. Set mass tolerance, which should be no less than the mass accuracy obtained experimentally.

   Typical mass accuracy values for external calibration should be ~100 to 300 ppm for delayed-extraction MALDI-TOF, and ~100 ppm for electrospray orthogonal TOF. For internal calibration, ~5 to ~20 ppm should be used for both methods. Typical mass accuracy of ~ 0.5 Da is appropriate when regular MALDI-TOF (i.e., without delayed extraction) is used with internal calibration.

3. Choose a minimum number of peptides required for matching a particular protein in the database to generate a hit.

   The default value is four, which can be changed depending on the search output and the total number of peptides submitted. If the number of search results is too large (i.e., >25), the minimum number of peptides required to match can be increased gradually to eliminate the number of false positives. In general, if the search results give more than five different proteins, and the number of hits exceeds 25 (except all the hits referring to one protein in different species), a false positive is present.

4. Choose a protein database to use for the search. Choose any database listed in the program, such as NCBInr, Genpept, Swiss Prot, Owl, and dbEST.

   NCBInr is recommended as the first choice for the search, because it combines most of the public domain protein databases (though dbEST is not included) to generate the largest nonredundant protein database, and because it is updated most frequently. Swiss Prot may also be chosen because it is the best annotated database.

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5. If the source of the protein of interest is known, select the species to perform a species-limited search. Alternatively, designate "all" for the species.

   In general, it is useful to designate "all," because it allows proteins occurring in different species with high homology to be found. Moreover, species prefiltering is imperfect because of poor usage of taxonomy (standard species naming conventions) in the databases. If the Latin taxonomic name for the species of interest is not known, see the NCBI Taxonomy Browser at http://www.ncbi.nlm.nih.gov/Taxonomy/tax.html.

6. Select the enzyme specificity and the number of "missed cleavage sites" to be employed.

   MS-Fit will search the peptide mass values submitted against calculated theoretical values of peptides cleaved by the specified enzyme. The termini of the matched peptides will be consistent with the cleavage specificity of the enzyme used to generate the peptide. Increasing the maximum number of allowed missed cleavages, enables matching to sequences with uncleaved sites internal to the peptide.

   If the digestion time employed is fairly long (>6 hr), it is possible (even likely) to find peptides present in the digest that are derived from nonspecific proteolytic cleavage. The option for employing the fictive enzyme "slymotrypsin" allows the occurrence of chymotryptic-like cleavages in tryptic digests. When using this choice it is important to increase the amount of missed cleavages allowed to three or more. It is also possible to combine the rules for two or more enzymes by adding options to the Enzyme item on the HTML form.

7. *Optional:* Select the molecular weight and pI range. Perform the first search with no molecular weight or pI restriction. Then adjust the range according to the search output.

   Because molecular weights from SDS-PAGE are not generally accurate, the range needs to be set at ±10 kDa. If the protein being analyzed is a processing or degradation product of a larger precursor, the molecular weight estimated from the gel would not be useful for the search.

8. Choose the desired types of modified residues to be searched.

   a. Specify modifications desired for cysteine residues to generate theoretical masses to match those cysteine-containing peptides that have been reduced and then alkylated with iodoacetic acid (carboxymethylation), iodoacetamide (carbamidomethylation), or 4-vinylpyridene (pyridylethylation).

   b. Specify additional chemical modifications of proteins that can be generated during PAGE, such as oxidation of methionine residues and acrylamide modification of cysteine residues.

   c. Specify peptide mass changes due to known posttranslational modifications, such as conversion of N-terminal glutamine to pyroglutamic acid, and phosphorylation of serine, threonine, and tyrosine.

   d. For any database entry with a methionine at the N terminus, specify whether the N-terminal peptide is to be considered in its original form or in a form where the methionine is removed and the penultimate amino acid is acetylated.

      If the database curators have removed the N-terminal methionine from the sequence, MS-Fit will not apply the acetylation modification.

Perform the search

9. Initiate the search. Two scoring systems are automatically displayed in search results: a modified MOWSE score (Pappin et al., 1993) and an MS-Fit rank. The higher the score, the higher number of peptide masses matched and the more likely the hit is of relevance. The output indicates the number of peptide masses matched and their corresponding sequences, and the matching protein's molecular weight, pI, accession number, species, and protein name. Click on the MS-digest index number to display

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the coverage of the matched peptides in the entire protein sequence. Click on the accession number to link to the NCBI Entrez.

A detailed description of the MOWSE and MS-Fit scoring system can be found by clicking on this parameter in the program.

Currently, MS-Fit employs only a simple ranking system. The results are sorted so that if multiple database entries are matched, the more likely matches are listed higher in the rank. All database entries matching the input data and parameters are ranked on the following basis: (1) database entries with the least number of unmatched masses are ranked higher, and (2) among equivalent matches (those with the same rank), the results are sorted in order of increasing index number.

10. To perform an alternative search in homology mode, select the box that allows for a single amino acid substitution per peptide.

    In this mode, the MS-Fit option for other possible modifications is by-passed. In practice, the homology mode should only be used when one or more of the following conditions applies: the peptide mass data have excellent mass accuracy (±10 ppm or better), a narrow intact protein molecular weight filter is used, and/or the hits will be saved and searched using MS-Tag (UNIT 16.6). The minimum amount of peptides with no amino acid substitution (usually set at one or two) controls the protein entry pulled from the database. The output displays the necessary single amino acid substitution and the corresponding sequence consistent with the experimental peptide mass data (not the sequence present in the database). These predictions need to be confirmed by MALDI post-source decay (MALDI-PSD) or tandem mass spectrometry.

11. *Optional:* To use one ProteinProspector search program as a prefilter for another search program, save the hits (index numbers for matching database entries) from the first program to a user-specified file. Retrieve this file using the second program, so that only those matching database entries are searched by the second program.

    Saved hits from MS-Fit can be used by other programs such as MS-Tag (UNIT 16.6) and MS-Edman.

COMMENTARY

Background Information

Initially, protein identification using peptide mass mapping was hampered mainly by the poor mass accuracy obtained using MALDI mass spectrometers, making unambiguous identification difficult. Recently, the development of MALDI-TOF equipped with delayed extraction has dramatically improved the mass resolution and mass accuracy (Vestal et al., 1995; Edmondson and Russell, 1996) at the subpicomole to femtomole protein levels. Moreover, the rapid growth of gene and protein databases resulting from large-scale genome sequencing projects of different organisms now offers significant power for mass spectrometric identification and characterization of proteins isolated from gels.

The advantage of the improved mass accuracy using delayed extraction is increased search specificity of each peptide, leading to a better discrimination of true hits versus false-positive search results (Clauser et al., unpub. observ.). Finally, peptide mass mapping is not only useful for identification of proteins existing in databases, but also for cross-species identification. This possibility relies on sequence conservation between two proteolytic cleavage sites (a stretch of ~10 to 15 amino acids) in homologous proteins of different organisms (Cordwell et al., 1997; Shevchenko et al., 1997).

Critical Parameters and Troubleshooting

Measuring masses as accurately as possible is the single most important factor in achieving the highest certainty of protein identification in a peptide mass fingerprinting experiment (K.R. Clauser, unpub. observ.). The more accurate the masses measured, the lower the mass tolerance is set, and the more unambiguous the results obtained.

Several steps must be followed to obtain reliable protein identification using peptide mass mapping, especially when dealing with cases yielding ambiguous search results. First,

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sort out all of the unassigned experimental peptide masses by attributing them to posttranslationally modified peptides (such as modification of cysteine, methionine, and glutamine; see Basic Protocol, step 8), peptides with nonspecific cleavage sites, enzyme autolysis products, adducts (sodium, potassium, and matrix adducts), matrix peaks, and contaminants (keratins from human and other species). Series of peaks with constant mass differences can be attributed to organic polymers, which may come from tubes or may have been added during the isolation or preparation procedures (Jungblut and Thiede, 1997). If there are still quite a few abundant peaks left unassigned by such means, perform a second database search using the set of unmatched mass values excluded from the first match hit, because more than one protein may be present in the gel spot due to comigration during gel electrophoresis (Jensen et al., 1996). Second, try another digest using a different enzyme, search both sets of data, and select the results that are common to both lists. The chance of obtaining a protein with a high score in both searches is extremely small (Jensen et al., 1996).

When no match is found in the protein databases, this may suggest that the gene encoding the protein of interest has not yet been sequenced or cloned. However, it may be known as an expressed sequence tag (EST) that represents part of the protein. Therefore, it may be useful to screen a dbEST database. Search times will typically be longer because of the multiframe translations and because the dbEST file is >3 times larger than the NCBInr file. When an EST database is selected, the number for DNA frame translation needs to be set. A user should select frame mode 6 unless it is known that the database being searched contains sequences exclusively cloned in one direction or contains known genes with sequences already in frame. Of course, a longer search time will be required when using frame mode 6 instead of 3. Users can also generate their own database for searching; the instructions are in the ProteinProspector home page. Finally, to obtain a protein identification unambiguously, in the authors' opinion, it is always prudent and in fact may be necessary to obtain a partial peptide sequence to choose correctly among possible MS-Fit search result possibilities, unless information from other experiments indicates what the protein could possibly be.

Anticipated Results

As an example, the use of MS-Fit for unambiguous protein identification is illustrated here. Figure 16.5.1 shows a spectrum obtained by delayed-extraction MALDI-reflectron-TOF mass spectrometry of an in-gel-digested protein spot from a two-dimensional gel. The peptide masses were externally calibrated using peptide standards. Twelve monoisotopic (Burlingame and Carr, 1996) peptide masses (Fig. 16.5.1) were submitted into the MS-Fit program. In this case, four modifications were considered: conversion of peptide N-terminal

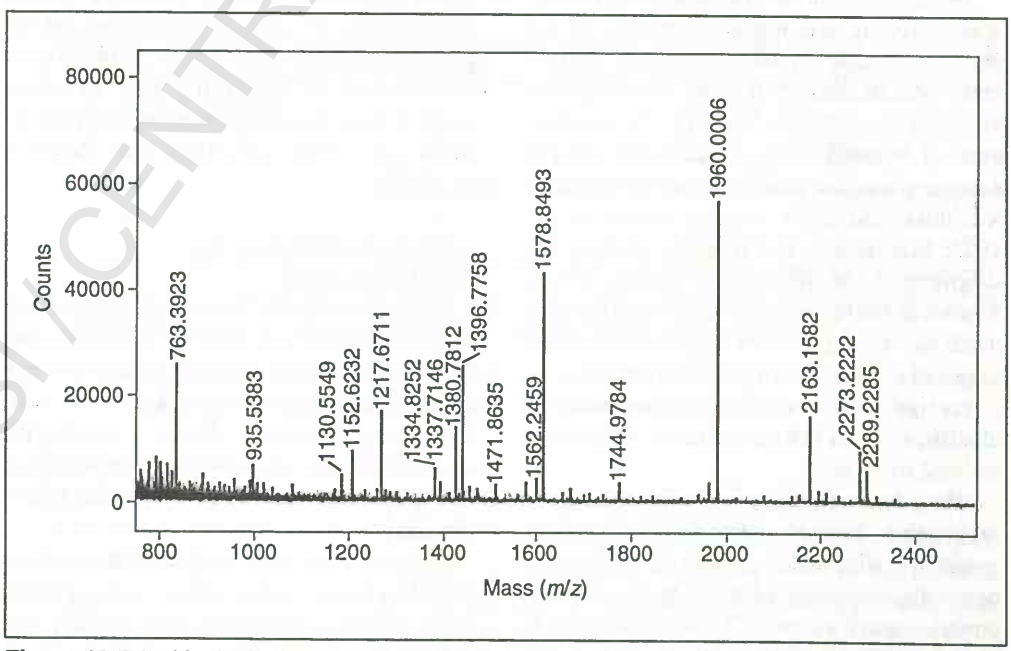

Figure 16.5.1 Mass spectrum obtained by delayed-extraction MALDI-reflectron-TOF mass spectrometry of an in-gel-digested protein spot from a two-dimensional gel.

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MS-Fit Search Results

Press stop on your browser if you wish to abort this MS-Fit search prematurely.
Sample ID (comment): **Spot #3, MALDI mass mapping**
Database searched: **NCBInr.06.09.98**
Molecular weight search **(1000-30000 Da)** selects **180441** entries.
Full pI range: **309620** entries.
Species search **(RATTUS NORVEGICUS)** selects **6834** entries.
Combined molecular weight, pI and species searches select **3032** entries.
MS-Fit search selects **1** entry.

Considered modifications:

| Peptide N-terminal Gln to pyroGlu | Oxidation of M | Protein N-terminus Acetylated |

Min. # Peptides to Match	Peptide Mass Tolerance (±)	Peptide Masses are	Digest Used	Max. # Missed Cleavages	Cysteines Modified by
4	**100.000 ppm**	**monoisotopic**	**Trypsin**	**1**	**acrylamide**

Peptide N terminus	Peptide C terminus	Input # Peptide Masses
Hydrogen (H)	**Free Acid (O H)**	**12**

Result Summary

Rank	MOWSE Score	# (%) Masses Matched	Protein MW (Da)/pI
1	9.71e+003	11/12 (91%)	28419.4 / 5.29

NCBInr.06.09.98 Accession #	Species	Protein Name
130860	RATTUS RATTUS	(D90258) proteasome subunit C8

Detailed Results

1. 11/12 matches (91%). 28419.4 Da, pI = 5.29. Acc. # 130860. RATTUS RATTUS. (D90258) proteasome subunit C8.

Data submitted	MH+ matched	Delta ppm	start	end	Peptide Sequence (Click for Fragment Ions)	Modifications
763.3923	763.4103	–23.5303	67	72	(R)LFNVDR(H)	
935.5383	935.5427	–4.6758	223	230	(K)GRHEIVPK(D)	
1130.5549	1130.5556	–0.6253	21	29	(R)VFQVEYAMK(A)	1 Met-ox
1152.6232	1152.6053	15.5002	101	110	(R)SNFGYNIPLK(H)	
1217.6711	1217.6490	18.1578	30	41	(K)AVENSSTAIGIR(C)	
1380.7812	1380.7422	28.2609	73	86	(R)HVGMAVAGLLADAR(S)	
1396.7758	1396.7371	27.7120	73	86	(R)HVGMAVAGLLADAR(S)	1 Met-ox
1471.8635	1471.8161	32.2353	197	208	(K)IIYIVHDEVKDK(A)	
1578.8493	1578.7876	39.0750	87	100	(R)SLADIAREEASNFR(S)	
1744.9784	1744.9135	37.2063	101	115	(R)SNFGYNIPLKHLADR(V)	
1960.0006	1959.8936	54.5998	1	20	(-)SSIGTGYDLSASTFSPDGR(V)	Acet N

1 unmatched mass: 1337.7

The matched peptides cover **43%** (110/255 AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 79534

Figure 16.5.2 MS-Fit search result. A database search was performed using the tryptic masses shown in Figure 16.5.1. A single protein was identified after restriction of the molecular weight range to 20 to 40 kDa and restriction of the species to rat. Reprinted with permission of the Regents of the University of California.

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glutamine to pyroglutamate, oxidation of methionine, acetylation of protein N-terminus, and carboxymethylation or acrylamide modification of cysteine. The peptide mass tolerance was chosen as 100 ppm because external calibration was used. The default values were used for maximum number of missed cleavage sites and minimum number for peptides to match (both set at 4). A first search was performed without selection of a particular species (enter "all") or molecular weight range (1000 to ~100,000 Da). In this unrestricted search, 182 entries were obtained in the search results, of which the three highest ranking proteins all indicated proteasome subunit C8 in human and rat, due to the high interspecies homology.

To reduce the number of false positives, the molecular weight range was set at 20 to ~30 kDa, as determined by SDS-PAGE. Alternatively, the minimum number of peptides matched may be increased for this purpose. Thirteen entries were displayed in the second search result, and the high-ranking proteins were the same as in the first search. At this point, it is fairly certain that the protein of interest is proteasome subunit C8. This conclusion was further supported in the third search result, in which the species searched was limited to rat. As shown in Figure 16.5.2, 91% of the peaks submitted were matched. Two molecular ions matched modified peptides containing oxidized methionine (1130.5549, 1396.7758) and N-terminal acetylation (1960.0006). Subsequently, the latter peptide with $[M + H]^{1+}$ at 1960.006 was analyzed further using MALDI-PSD. This 42-Da shift in spectrum established the presence of N-terminal acetylation by observation of the appropriate ion sequence. When operating in reflectron mode, peptides containing oxidized methionine residues usually eliminate 64 u (mass units; $-SOCH_4$) as a neutral moiety, and the resulting metastable fragment ion is not focused well and thus typically shows a 62-Da loss from the parent ion. The peak at 1334.8252 represents a fragment ion from peptide of $[M + H]^{1+}$ at 1396.7758.

In conclusion, this protein can be successfully identified as rat proteasome subunit C8 using the MS-Fit program. However, in general, to ascertain reliable protein identification, it is always advisable to confirm such MS-Fit results with independent peptide sequence data using either MALDI-PSD or MALDI collision-induced dissociation (MALDI-CID).

Time Considerations

Search times depend on the database selected (also see Critical Parameters and Troubleshooting) and on the quality of data submitted (e.g., the amount of tryptic peptides, the mass accuracy). They can vary from less than 1 min to 15 min or more.

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Internet Resources

http://prospector.ucsf.edu

University of California's ProteinProspector site.

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Searching Sequence Databases Over the Internet: Protein Identification Using MS-Tag

Although peptide mass mapping is fast and simple, its success can be compromised by the purity of the protein in one gel spot, errors in the sequence database, and the detection of too few peptides in the MS map of a given protein to permit reliable database matching. In those cases, partial amino acid sequence that is determined using post-source decay (PSD) analysis or tandem mass spectrometry is required to establish the correct protein identification among possibilities suggested by MS-Fit (*UNIT 16.5*).

Post-source decay (Kaufmann et al., 1993, 1994) involves the detection of the fragmentation product ions (metastable ions) of a selected mass value window containing the precursor ion chosen, that occur in the field-free region between the ion source and the reflectron. For a discussion of the basic principle of MALDI-PSD-MS for peptide sequencing, refer to *UNIT 16.1*. This unit covers some of the practical issues in obtaining and analyzing a typical PSD spectrum. The Basic Protocol introduces MS-Tag (see *http://prospector.ucsf.edu*), a database interrogation program that enables the identification of proteins with a limited number of peptide fragment ions by matching existing database sequences. Although a typical PSD spectrum will usually not provide enough sequence ions for de novo manual sequence interpretation (particularly in the higher mass range, e.g., >500 to 800 Da), these ions together with the parent ion are statistically much better identifiers in database searching than only the molecular weights of tryptic peptides. Unlike peptide fingerprinting, database searching based on fragment ion information is often able to identify a single peptide sequence in the database that matches all the input information.

Several computer programs employing fragment ion information to search databases are available on the internet (see *http://donatello.ucsf.edu/onramp.html* and sites listed therein). Among them, MS-Tag is a user-friendly program that simultaneously considers a variety of ion types, but still allows single-mismatch-tolerant searching. However, the authors suggest that a frequent user of MS-Tag should be able to interpret sequences from those spectra that do contain sufficient information to enable de novo interpretation.

Although MALDI-PSD database searching is a widely used tool, an alternative method is analysis by MALDI collision-induced dissociation (MALDI-CID). This is presented in the Alternate Protocol.

DATABASE SEARCHING WITH MS-TAG

BASIC PROTOCOL

The basic principles of MS-Tag are detailed in the instruction section of the Web site (*http://prospector.ucsf.edu*). Users can find explanations for every input parameter by just clicking on the highlighted terms.

1. Type in the mass and charge state of the parent ion (masses detected in MS mode are preferred).
2. Define the parent ion mass tolerance.

   The tolerance range will depend on the instrument.
3. Input all fragment ions and define their mass tolerance, which is usually higher than the parent ion mass tolerance.

   Ions with higher intensities are preferred if a number of ions are available.

Contributed by C.R. Jiménez, L. Huang, Y. Qiu, and A.L. Burlingame

Current Protocols in Protein Science (1998) 16.6.1-16.6.7

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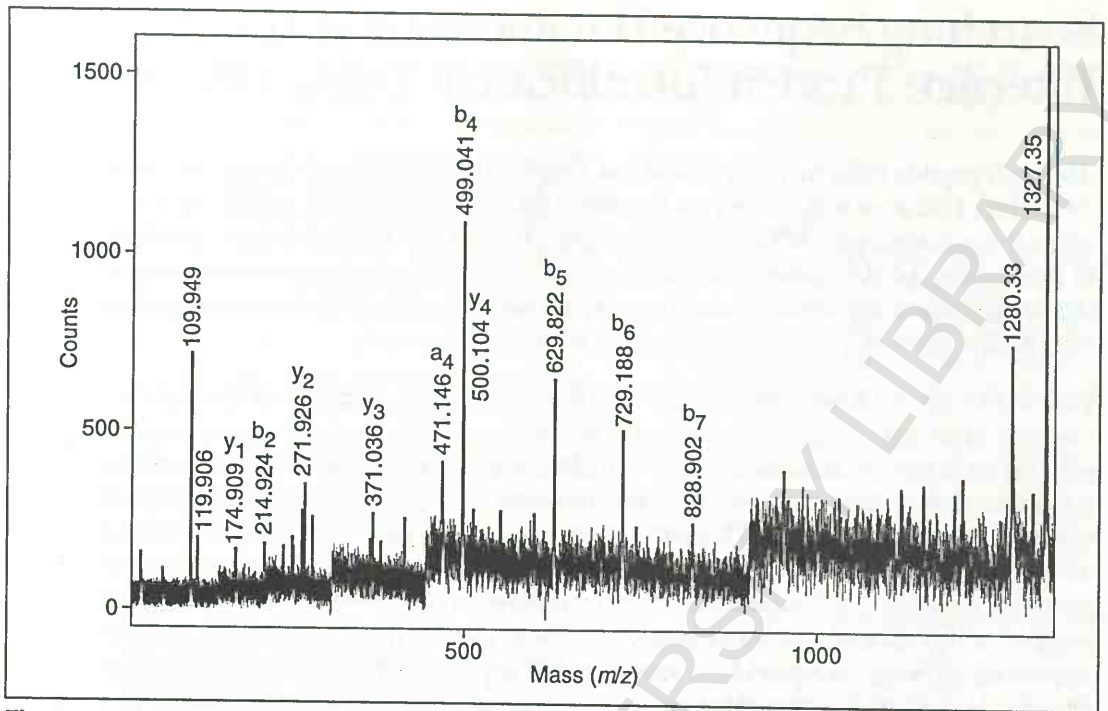

Figure 16.6.1 PSD spectrum of a tryptic peptide of an unknown protein isolated from mouse liver. This peptide has sequence homology to bovine inorganic pyrophosphatase (DVFMVVEVPR), where one mutation is found (from E1 to D1).

4. Specify the type of input masses (e.g., all monoisotopic, all average, or parent ion is monoisotopic and fragment ions are average).

5. Specify the database searched and the type of frame translation if a nucleotide database is selected.

   The ability to search against MS-Fit hits is particularly useful when the target protein is not in current protein databases but is included in the expressed sequence tag (EST) database. The EST databases are currently much larger than other databases. Therefore, direct MS-Tag searching against an EST database is statistically much more demanding and may fail due to the much longer search time.

6. Specify the species from which the protein of interest is isolated, the protein molecular weight range, the type of enzyme used for protein digestion, the type of cysteine modification, and the maximum unmatched fragment ions. Default parameters may be used for other choices listed on the Web page.

7. Specify the search mode as identity or homology. Search in identity mode first; if not successful, then search in homology mode. Begin search.

   Typical results are depicted in Figure 16.6.1 and Figure 16.6.2, and are discussed in Anticipated Results.

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MS-Tag Search Results

Press stop on your browser if you wish to abort this MS-Tag search prematurely.
Sample ID (comment): **Apo A-1 1040 AKPVLEDLR**
Database searched: **NCBInr.05.04.98**
Molecular weight search **(1000 - 100000 Da)** selects **287683** entries.
Number of sequences passing through parent mass filter: **150221**
MS-Tag search selects **1** entries.
Ion Types Considered: **a b B y n h l**

Search Mode	Max. # Unmatched Ions	Peptide Masses are	Digest Used	Max. # Missed Cleavages
homology	**0**	**monoisotopic**	**Trypsin**	**1**

Cysteines Modified by	Peptide N terminus	Peptide C terminus
acrylamide	**Hydrogen (H)**	**Free Acid (O H)**

Parent mass: **1327.6000 (±0.6000 Da)**
Fragment Ions used in search: **174.9, 255.0, 268.9, 271.9, 284.9, 371.0, 415.9, 471.1, 499.0, 629.8, 729.2, 828.9 (±1.20 Da)**
Parent mass shift: **±15.0 Da**
Composition Ions present: **HF**

Result Summary

Rank	MS-Digest Index #	NCBInr.05.04.98 Accession #	Protein MW (Da)	Species	Calculated MH+ (Da)	MH+ Error (Da)
1	**101341**	**585322**	**32844.4**	**BOVIN**	**1341.6989**	**–14.0989**

Modification	Sequence
E1->D	**(K)DVFHMVVEVPR(W)**

Protein Name
INORGANIC PYROPHOSPHATASE (PYROPHOPHATE PHOSPHO-HYDROLASE) (PPASE)
gil539751|pir||A45153 inorganic pyrophosphatase (EC 3.6.1.1) - bovine

Detailed Results

Rank	MS-Digest Index #	NCBInr.05.04.98 Accession #	Protein MW (Da)	Species	Calculated MH+ (Da)	MH +Error (Da)
1	**101341**	**585322**	**32844.4**	**BOVIN**	**1341.6989**	**–14.0989**

Modification	Sequence
E1->D	**(K)DVFHMVVEVPR(W)**

Protein Name
INORGANIC PYROPHOSPHATASE (PYROPHOPHATE PHOSPHO-HYDROLASE) (PPASE)
gil539751|pir||A45153 inorganic pyrophosphatase (EC 3.6.1.1) - bovine

Fragment-Ion (m/z)	174.9	255.0	268.9	271.9	284.9	371.0	415.9	471.1	499.0	629.8	729.2	828.9
Ion-type Delta Da	y_1 –0.22	y_2-NH3 –0.15	HM –0.21	y_2 –0.27	FH –0.24	y_3 –0.24	FMH –0.28	a_4 –0.14	b_4 –0.23	b_5 –0.47	b_6 –0.14	b_7 –0.49 y_7 –0.56

Figure 16.6.2 MS-Tag search result. An MS-Tag search of the NCBInr database in homology mode with the PSD ions of the tryptic peptide shown in Figure 16.6.1 yields one single peptide sequence (DVFHMVVEVPR). Reprinted with permission from the University of California, San Francisco, Mass Spectrometry Facility.

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SEQUENCE ANALYSIS USING MALDI-CID

Although MALDI-PSD combined with database searching (see Basic Protocol) has emerged as the primary approach for identifying gel-separated proteins, limitations exist. For example, due to the nature of the gating device, the resolution of a precursor ion selection cannot exceed ~100 (M/ΔM) without serious loss of sensitivity. This value would barely enable the distinction between a parent ion at *m/z* 2000 and its water-loss ion. More importantly, the performance of PSD tends to be unpredictably dependent on the amino acid composition of individual peptides. This may be avoided by relying on high-energy MALDI collision-induced dissociation (MALDI-CID), because the use of collision gas with current reflectron instruments usually increases the intensities of product ions in the low mass region only (mainly immonium ions; Stimson et al., 1997). Often complete sequence ion information is needed to determine the sequence of peptides de novo (particularly important if the protein of interest is not represented in any current databases) or in studies of the structure of posttranslational and xenobiotic modifications. In many cases, these problems may be addressed by high-energy MALDI-CID.

High-energy MALDI-CID is often performed on hybrid instruments, such as a tandem double-focusing magnetic orthogonal-acceleration time-of-flight (TOF) mass spectrometer (Bateman et al., 1995; Medzihradszky et al., 1996, 1997). The use of a double–focusing sector mass analyzer (such as MS1, a mass analyzer for separating parent ions using appropriate electric fields, and in combination with magnetic fields) enables the selection of a monoisotopic precursor ion with mass up to *m/z* 2000. The ions selected by MS1 then collide with xenon gas to generate a variety of ion types, which are then accelerated into a linear TOF analyzer orthogonal to the original direction of travel. The sampling frequency for this mode of operation is 100%. The signal-to-noise ratio in a MALDI-CID spectrum is far superior to that of a PSD spectrum. More importantly, many ion types that are unique to the high-energy process (e.g., d, v, and w ions) can be observed in a high-energy MALDI-CID spectrum but not in a PSD spectrum (which is very similar to a low-energy CID process). These high-energy ions have considerable value in the de novo determination of complete and unambiguous amino acid sequence (Medzihradszky and Burlingame, 1994), such as discrimination of leucine and isoleucine residues (Biemann, 1990). Therefore, MALDI-CID holds the promise to characterize unknown proteins, particularly those not represented in any current database, and can more explicitly determine the sites of posttranslational or xenobiotic modifications on target proteins.

For example, Figure 16.6.3 shows a MALDI-CID spectrum of a tryptic peptide of human serum albumin (HPYFYAPELLFFAK*R, *m/z* 2360.5) that is modified by benoxaprofen glucuronide (Qiu et al., 1998), as indicated by the asterisk in the sequence above. As benoxaprofen contains a chlorine atom, the $^{12}C^{35}Cl$ isobar for the precursor mass was selected by MS1 for CID analysis. In this spectrum, all C-terminal sequence ions (y ions) together with their corresponding high-energy ions (v and w ions) allow the unambiguous determination of both the sequence and the site of modification of this peptide. The 461-unit mass shift of all y ions except y_1 indicates the benoxaprofen glucuronide modification on the lysine residue near the C terminus. The presence of w_6 (*m/z* 1183.5) and w_7 (*m/z* 1296.6) distinguishes leucines from isoleucines (for isoleucines, w_6 and w_7 should be at *m/z* 1197.5 and 1310.6, respectively). The N-terminal sequence ions (b ions), internal fragments, and fragments from the drug moiety also aid or confirm the final data interpretation.

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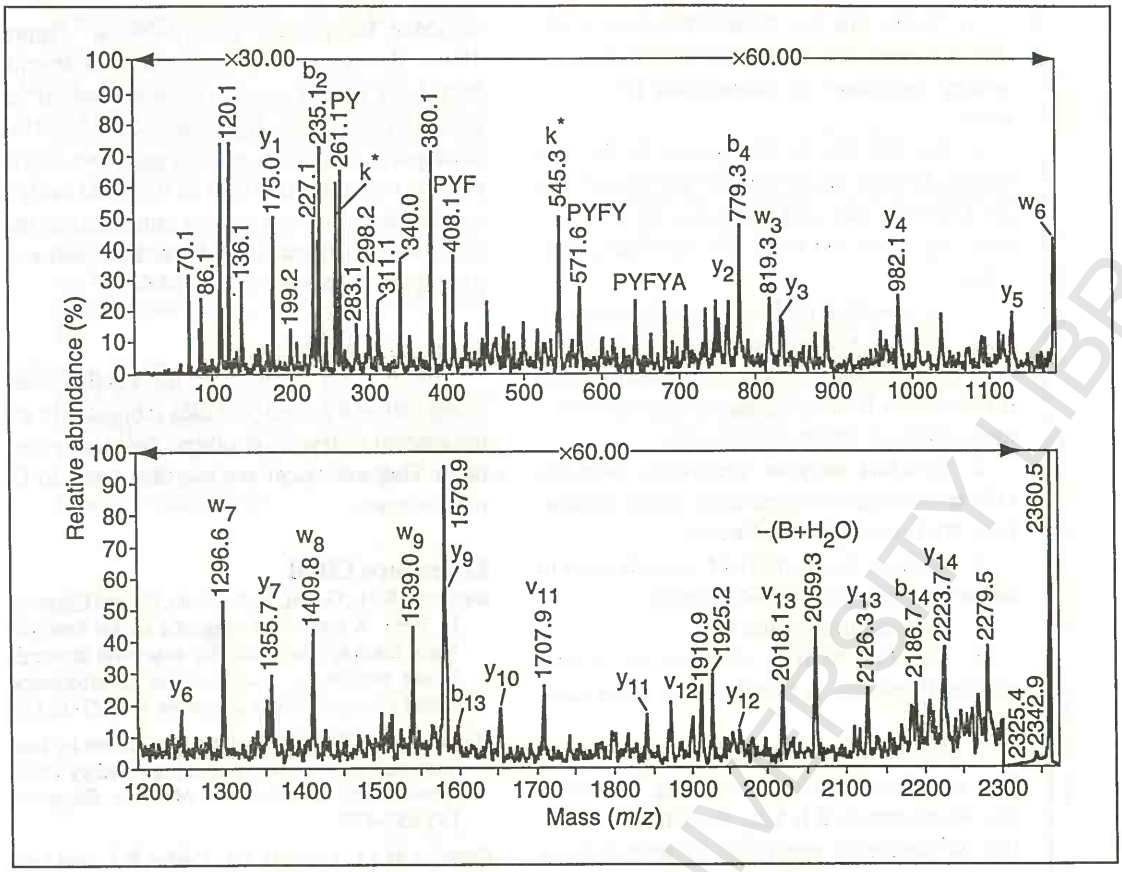

Figure 16.6.3 MALDI-CID spectrum of a tryptic peptide of human serum albumin (amino acids 146 to 160; HPYFYAPELLFFAK*R; *m/z* 2360.5). Asterisk indicates modification with benoxaprofen glucuronide.

COMMENTARY

Background Information

In MALDI-TOF-MS spectra obtained in a reflectron mode, most of the fragment ions (metastable) formed in the field-free region cannot be observed because they are not in energy focus. However, fragment ions with masses close to the mass value of the parent ion can still be observed, albeit with both lower resolution and mass accuracy. To focus these metastable fragment ions throughout the entire mass range, the reflectron voltage needs to be successively stepped down to record approximately nine to fourteen consecutive mass scale segments, depending on the mass of the parent ion. These partially overlapping mass segments are then mass resolved to an accuracy of ±0.3 Da, and can be calibrated and stitched together to form a full PSD mass spectrum. One may still find that fragment ion peaks with different masses recorded in one segment have different mass accuracy (sometimes the difference can be as high as 0.3 Da within a segment). Typically, ions with higher masses will have better mass accuracy than those with lower masses because they are better focused by the reflectron. More recently introduced curved-field reflectrons (Cordero et al., 1995) focus all the ions into a small region near the exit of the reflectron. This new feature enables approximately ten times faster acquisition of the product ion spectrum, because it can simultaneously collect the entire PSD mass spectrum without changing the reflectron voltage.

Critical Parameters and Troubleshooting

After submitting a search request, oftentimes no match is retrieved for a given set of input parameters, and the MS-Tag program suggests decreasing the specificity of the input parameters. Because there are many restrictions that can be changed, it may be difficult for a new user to pinpoint the critical parameters that prevent the search from retrieving hits. In this situation, the following troubleshooting suggestions may save time.

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1. Verify that the mass calibration of the data is correct. Mass accuracy of the parent ion is very important for minimizing false positives.

2. Do not specify the species in the first search, because some species may have different names in different databases. Specify species only when too many hits have been identified.

3. Do not limit molecular weight range.

4. Provide a broad enough mass tolerance window for fragment ions. Fragment ions are differentially focused by the reflectron, so their mass accuracy varies significantly.

5. Deselect enzyme specificity. Nonspecific enzymatic cleavages often occur, particularly after overnight incubations.

6. Increase the number of ions allowed to be unmatched when no hit is found.

7. Deselect immonium ions.

8. Use the NCBInr database as a first choice. If nothing is found, search other databases.

If none of these changes results in search hits, switch to homology searching. In homology search mode, it is very important to maintain the maximum specificity of input data, or too many false positives may be generated. Additionally, only peptides with one amino acid difference from a peptide sequence existing in the database can be retrieved. If two mutations occur in your peptide, no hit will be found. For peptides containing oxidized methionine, homology mode must be used. In this case, the parent mass shift can be limited to ±17 Da.

Anticipated Results

PSD spectra are usually acquired at a laser power well above the threshhold required for observation of ions corresponding to the intact analytes (Riahi et al., 1994). Whether this enhancement of internal (vibronic) energy is due to the desorption of parent ions with deposition of higher internal energy or to the increased collision number resulting from a larger matrix plume volume is currently not known. The particular results obtained during a PSD experiment are also dependent on the properties of the particular matrices used (see MALDI sample preparation, *UNIT 16.3*; Stimson et al., 1997). For PSD analysis of peptides, α-cyano-4-hydroxy-*trans*-cinnamic acid (HCCA) is the matrix of choice because it seems to promote ion fragmentation more than other matrices.

The following example illustrates the use of PSD combined with MS-Tag searching of the database for protein identification. Figure 16.6.1 shows the PSD spectrum of a tryptic peptide of an unknown protein isolated from mouse liver. An MS-Tag search of the NCBInr database in homology mode yields one single peptide sequence (DVFHMVVEVPR) with a single amino acid mismatch, compared to the sequence of a tryptic peptide from bovine inorganic pyrophosphatase (Fig. 16.6.2).

Time Considerations

Search times depend on the database selected and on the quality of data submitted (e.g., the amount of tryptic peptides, the mass accuracy). They can vary from less than 1 min to 15 min or more.

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Internet Resources

http://prospector.ucsf.edu

University of California's ProteinProspector site.

http://donatello.ucsf.edu/onramp.html

University of California site for programs employing fragment ion information to search databases (also see sites listed therein).

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PREPARATION AND HANDLING OF PEPTIDES

18

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IDENTIFICATION OF PROTEIN INTERACTIONS

19

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CHAPTER 19
Identification of Protein Interactions

INTRODUCTION

Protein interactions are intrinsic and vital to many cellular processes. In recent years many new techniques have been developed for the study of this important topic; the review by Phizicky and Fields (1995) provides an excellent general overview of this subject. This chapter describes biochemical, physical, and genetic methods that are used to detect specific protein interactions. A companion chapter in preparation will present a series of complementary methods that are used to provide quantitative information about protein interactions, such as the strength and kinetics of binding.

This chapter begins with brief overview of the analysis of protein interactions in which some of the basic kinetic and thermodynamic concepts are described (*UNIT 19.1*). This is followed by a presentation of one of the most widely used approaches for detecting protein interactions, the yeast two-hybrid/interaction trap system (*UNIT 19.2*). In this genetic method transcriptional activity is used as a measure of protein interaction.

Future plans for this chapter include units on a phage-based expression cloning system used to identify interacting proteins, and surface plasmon resonance (SPR), another useful method for discovery and detection of protein interactions that is gaining in popularity.

LITERATURE CITED

Phizicky, E.M. and Fields, S. 1995. Protein-protein interactions: Methods for detection and analysis. *Microbiol. Rev.* 59: 94-123.

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Analysis of Protein-Protein Interactions

BASIC CONCEPTS

Most of the work of living organisms is performed by proteins. Proteins do their work by acting on other macromolecules: nucleic acids, carbohydrates, lipids, and, especially, other proteins. Inside cells, protein-protein interactions are instrumental in enzymatic actions upon protein substrates and in the fleeting and lasting protein assemblies that govern signal transduction, cell division, DNA replication, and transcription initiation. Outside cells, protein interactions allow cells to talk with each other: ligands expressed on the cell surface often bind protein receptors expressed on adjacent cells, and secreted protein ligands bind receptors on distant cells. Techniques to detect and study protein-protein interactions have become steadily more easy to use in recent years. This unit, and those that follow, lay them out.

Qualitative statements about protein-protein interactions inform much of the current biological literature. This unit presents the basics for understanding such qualitative statements—i.e., the quantitative concepts by which these can be evaluated. Colloquially, protein interactions are often referred to as either **stable** or **transient**. The statement that a certain interaction is **stable** is typically used when the interacting proteins form a complex. Membership in a protein complex is most commonly defined operationally—based on detection of the protein after its coprecipitation with a reagent specific for another member of the complex—but is sometimes defined based on a strong and biologically plausible interaction in a two-hybrid experiment. Proteins that form such complexes with one another are often referred to as **partners**. By contrast, the term **transient** is used to denote, e.g., interactions between an enzyme and a protein substrate.

As the preceding example suggests, there is no particular correlation between the stability of a protein interaction and its biological significance. It is also worth noting that there are exceptions to commonly held assumptions about stable and transient interactions: some interactions observed in coprecipitation experiments have no biological validity, whereas some enzyme-substrate interactions last for minutes. For these reasons, it is often useful to think of protein interactions in more formal terms. The strength, or **affinity**, of an interaction between two proteins is described by **equilibrium parameters**. The speed at which an interaction occurs, and at which interacting proteins come apart, is given by **kinetic parameters**.

EQUILIBRIUM PARAMETERS

The strength of an interaction can be given as the **equilibrium dissociation constant**, K_d, or by its reciprocal, K_a (discussed below). When two proteins A and B associate, the K_d is given by

$$K_d = \frac{[A][B]}{[AB]}$$

where [AB] is the concentration of the complexed species and [A] and [B] are the concentrations of the noncomplexed species. Concentrations are given in molar terms, as is the K_d.

There are two useful special cases to keep in mind. First, when A and B are present in equal concentration, the concentration at which half of each protein is present in the form of AB complex is the K_d. Second, when the concentration of A vastly exceeds the

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Table 19.1.1 Dissociation Constants for Some Biologically Significant Interactions

Interaction	K_d
Streptavidin-biotin binding	10^{-14} M
Antibody-antigen interaction with good antibody	10^{-8} to 10^{-10} M
DNA binding protein with specific site	10^{-8} to 10^{-10} M
Antibody-antigen interaction with weak antibody	10^{-6} M
Enzyme-substrate interations	10^{-4} to 10^{-10} M
Cooperative interaction between DNA-bound phage repressors	10^{-4} M
Cooperative interaction between phage repressor and *E. coli* RNA polymerase	10^{-2} M

concentration of B (say, by 100-fold)—typically indicated [A] >> [B]—the concentration of A at which half of the B is found in the AB complex approximates K_d.

Typical K_d values for biologically significant interactions are given in Table 19.1.1. Note that many significant interactions are weak.

Alternatively, affinity may be given as an **equilibrium association constant**, K_a. The K_a is simply

$$K_a = \frac{[AB]}{[A][B]}$$

In other words, it is the reciprocal of the equilibrium dissociation constant: $K_a = 1/K_d$. Association constants are used less often than K_d, but do appear in the literature of some subfields—for example, in descriptions of antibody-antigen interactions.

The strength of an interaction is directly proportional to the change in Gibbs free energy (ΔG) when A and B interact, which is given by

$$\Delta G = \Delta H - T\Delta S$$

where T is the temperature in degrees Kelvin, ΔS is the change in entropy (S), and ΔH is the change in enthalpy (H). ΔG is given in units of kilocalories/mole (kcal mol^{-1}). An increase in the free energy of an interaction by 1 kcal decreases the K_d by a factor of ~7. Note that the temperature affects the entropy term of the equation. As the temperature decreases, reactions driven by enthalpy are less affected by losses in entropy, and are usually favored. By contrast, reactions whose free energy is derived largely from favorable changes in entropy are disfavored at lower temperatures. This means that the dependence of a protein association on temperature can often provide a clue to which term predominately contributes to the free energy change involved in that association.

The relationship between K_a and ΔG is defined as follows:

$$\Delta G^0 = -RT \ln \frac{[AB]}{[A][B]}$$

$$\Delta G^0 = -RT \ln K_a = -RT \ln\left(\frac{1}{K_d}\right) = -RT \ln K_d$$

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where ΔG^0 is the free energy change under standard conditions (25°C); R is the universal gas constant (1.9872 calmole^{-1}K^{-1}); and T is the temperature in degrees Kelvin (25°C is 289.1 K). Therefore,

$$\Delta G^0 = 0.588 \ln K_d$$

and, since $\ln x = 2.303 \log_{10} x$, $\Delta G^0 = 1.36 \log_{10} K_d$. For example:

$$\text{(a) } K_d = 1 \times 10^{-14}, \text{ then } \Delta G^0 = -19.04 \text{ kcal mol}^{-1}$$

$$\text{(b) } K_d = 1 \times 10^{-2}, \text{ then } \Delta G^0 = -2.72 \text{ kcal mol}^{-1}$$

The strepavidin-biotin reaction (a) is intrinsically more favorable in the direction of binding, [AB] formation, than the low-affinity interaction (b) involving phage repressor (see Table 19.1.1). It should be pointed out that the DG values calculated above assume that the molar ratio of reactants [A] [B] and product [AB] is 1 M (standard state).

Many (but by no means all) biologically important protein interactions seem to be largely driven by ΔH, or changes in enthalpy. That is fortunate, in that changes in entropy on binding are very hard to quantitate, or even think about precisely. For example, proteins in solution are surrounded by water molecules that form hydrogen bonds with surface residues. When two proteins interact, the ordered arrangement of the water molecules that surrounded the interacting surfaces of the proteins is often disrupted, and this loss of order provides an entropically favorable term to the free energy of the interaction. Such changes in the free energy due to entropy are very hard to predict. By contrast, enthalpic changes are easier to understand. If formation of one hydrogen bond liberates about –1 kcal M^{-1}, and formation of a particular ionic contact liberates about –2 kcal M^{-1}, then the energies of these changes are additive such that the formation of both bonds usually liberates –3 kcal M^{-1}—which means that the K_d is decreased >100-fold by the enthalpic changes.

Kinetic Parameters

The above descriptions of protein interactions make no reference to the speed at which association or dissociation occurs. These speeds are given by **kinetic parameters**.

The **dissociation rate constant**, k_{dissoc}, gives the speed at which the AB complex dissociates into A and B (AB → A + B). k_{dissoc} is a first-order rate constant—i.e., one that is dependent on the concentration of one species, in this case the AB complex—and is given by the rate of decrease in the concentration of AB:

$$k_{dissoc}[A][B] = \frac{-d[AB]}{dt}$$

Its units are those of reciprocal time (t in the equation), usually given in sec^{-1}. For example, a dissociation rate constant of 10^{-4} sec^{-1} means that one in 10^4 of the AB complexes present comes apart each second.

Similarly, the **association rate constant**, k_{assoc}, gives the speed at which A and B associate to form an AB complex (A + B → AB). This is a second-order reaction—i.e., its speed depends on the concentrations of both A and B—and its rate constant is is given by

$$k_{assoc}[A][B] = \frac{+d[AB]}{dt}$$

Its units are those of reciprocal concentration × reciprocal time, typically given in M^{-1} sec^{-1}.

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For example, suppose that protein A is present at a concentration of 10^{-6} M in a cell, that protein B is injected to a nuclear concentration of 10^{-5} M, and that the rate constant for this antibody-antigen association is 10^{-4} M^{-1} sec^{-1}. After injection, the concentration of AB will be

$$[AB] = [A] \times [B] \times K_{assoc}$$

$$= (10^{-6}\,M)(10^{-5}\,M)(10^{-4}\,M)$$

$$= 10^{-7}\ M\ sec^{-1}$$

That is, in the first second after mixing, 10^{-7} M of AB complex will form. Every 10-fold increase in the concentration of either reactant increases the rate of product formation 10-fold.

Note that, at equilibrium, by definition,

$$\frac{d[AB]}{dt} = 0$$

and, therefore, for the AB interaction,

$$K_d = \frac{k_{dissoc}}{k_{assoc}}$$

The fact that the strength of equilibrium interactions reflects the speed of associations and dissociations has important consequences. To understand this, imagine two pairs of proteins that interact with the same K_d. Proteins A and B come together slowly, but, once the AB complex forms, it takes a long time to come apart. By contrast, proteins C and D come together rapidly, and the CD complex dissociates rapidly. There are two common cases in which these kinetic differences in the AB and CD associations would be significant.

One is in measurement. Many techniques, such as the "pulldown" and immunological coprecipitation techniques described in this chapter, rely on the the fact that proteins remain associated during some sequence of steps, while they are being separated from other proteins in a mixture and while the isolated complex is being rinsed. No matter how tightly the proteins associate, if they come apart before their complex can be separated and rinsed, the complex will not be detected. Moreover, if the AB and CD interactions have the same K_d, but CD comes apart more rapidly, then a coprecipitation experiment can falsely suggest that the CD association is weaker.

The second case concerns the biological effects. Many biological phenomena, such as the transcription phenotypes resulting from protein-protein interactions in two-hybrid experiments (*UNIT 19.3*), seem to be well-described by consideration of equilibrium measurements. However, it is worth keeping in mind that any biological process that results from the association of two proteins requires a minimum time to occur. For some enzyme-substrate interactions, the minimum time may be on the order of microseconds, but for others, such as the initiation of DNA replication, it may be measured in seconds. If the complex dissociates faster than the minimum time, then, no matter how tight the interaction, the process will not occur. If AB and CD have the same K_d, but CD dissociates faster, the association may not produce a biological effect.

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Interaction Trap/Two-Hybrid System to Identify Interacting Proteins

To understand the function of a particular protein, it is often useful to identify other proteins with which it associates. This can be done by a selection or screen in which novel proteins that specifically interact with a target protein of interest are isolated from a library. One particularly useful approach to detect novel interacting proteins—the two-hybrid system or interaction trap (see Figs. 19.2.1 and 19.2.2)—uses yeast as a "test tube" and transcriptional activation of a reporter system to identify associating proteins (see Background Information). This approach can also be used specifically to test complex formation between two proteins for which there is a prior reason to expect an interaction.

In the basic version of this method (see Fig. 19.2.2), the plasmid pEG202 or a related vector (see Fig. 19.2.3 and Table 19.2.1) is used to express the probe or "bait" protein as a fusion to the heterologous DNA-binding protein LexA. Many proteins, including transcription factors, kinases, and phosphatases, have been successfully used as bait proteins. The major requirements for the bait protein are that it should not be actively excluded from the yeast nucleus, and it should not possess an intrinsic ability to strongly activate transcription. The plasmid expressing the LexA-fused bait protein (see Table 19.2.1) is used to transform yeast possessing a dual reporter system responsive to transcriptional activation through the *LexA* operator. In one such example, the yeast strain EGY48 (see Table 19.2.2) contains the reporter plasmid pSH18-34. In this case, binding sites for LexA are located upstream of two reporter genes. In the EGY48 strain, the upstream activating sequences of the chromosomal *LEU2* gene—required in the biosynthetic pathway for leucine (Leu)—are replaced with *LexA* operators (DNA binding sites). pSH18-34 contains a *LexA* operator–*lacZ* fusion gene. These two reporters allow selection for transcriptional activation by permitting selection for viability when cells are plated on medium lacking Leu, and discrimination based on color when the yeast is grown on medium containing Xgal (*APPENDIX 4A*).

In Basic Protocol 1, EGY48/pSH18-34 transformed with a bait is characterized for its ability to express protein (Support Protocol 1), growth on medium lacking Leu, and for the level of transcriptional activation of *lacZ* (see Fig. 19.2.2A). A number of alternative strains, plasmids, and strategies are presented which can be employed if a bait proves to have an unacceptably high level of background transcriptional activation.

In an interactor hunt (Basic Protocol 2), the strain EGY48/pSH18-34 containing the bait expression plasmid is transformed (along with carrier DNA made as described in Support Protocol 2) with a conditionally expressed library made in the vector pJG4-5 (see Fig. 19.2.6 and Table 19.2.3). This library uses the inducible yeast *GAL1* promoter to express proteins as fusions to an acidic domain ("acid blob") that functions as a portable transcriptional activation motif (act) and to other useful moieties. Expression of library-encoded proteins is induced by plating transformants on medium containing galactose (Gal), so yeast cells containing library proteins that do not interact specifically with the bait protein will fail to grow in the absence of Leu (see Fig. 19.2.2B). Yeast cells containing library proteins that interact with the bait protein will form colonies within 2 to 5 days, and the colonies will turn blue when the cells are streaked on medium containing Xgal (see Fig. 19.2.2C). The DNA from interaction trap positive colonies can be analyzed by polymerase chain reaction (PCR) to streamline screening and detect redundant clones in cases where many positives are obtained in screening (see Alternate Protocol 1). The plasmids are isolated and characterized by a series of tests to confirm specificity of the interaction with the initial bait protein (Support Protocols 3 to 5). Those found to be specific are ready for further analysis (e.g., sequencing).

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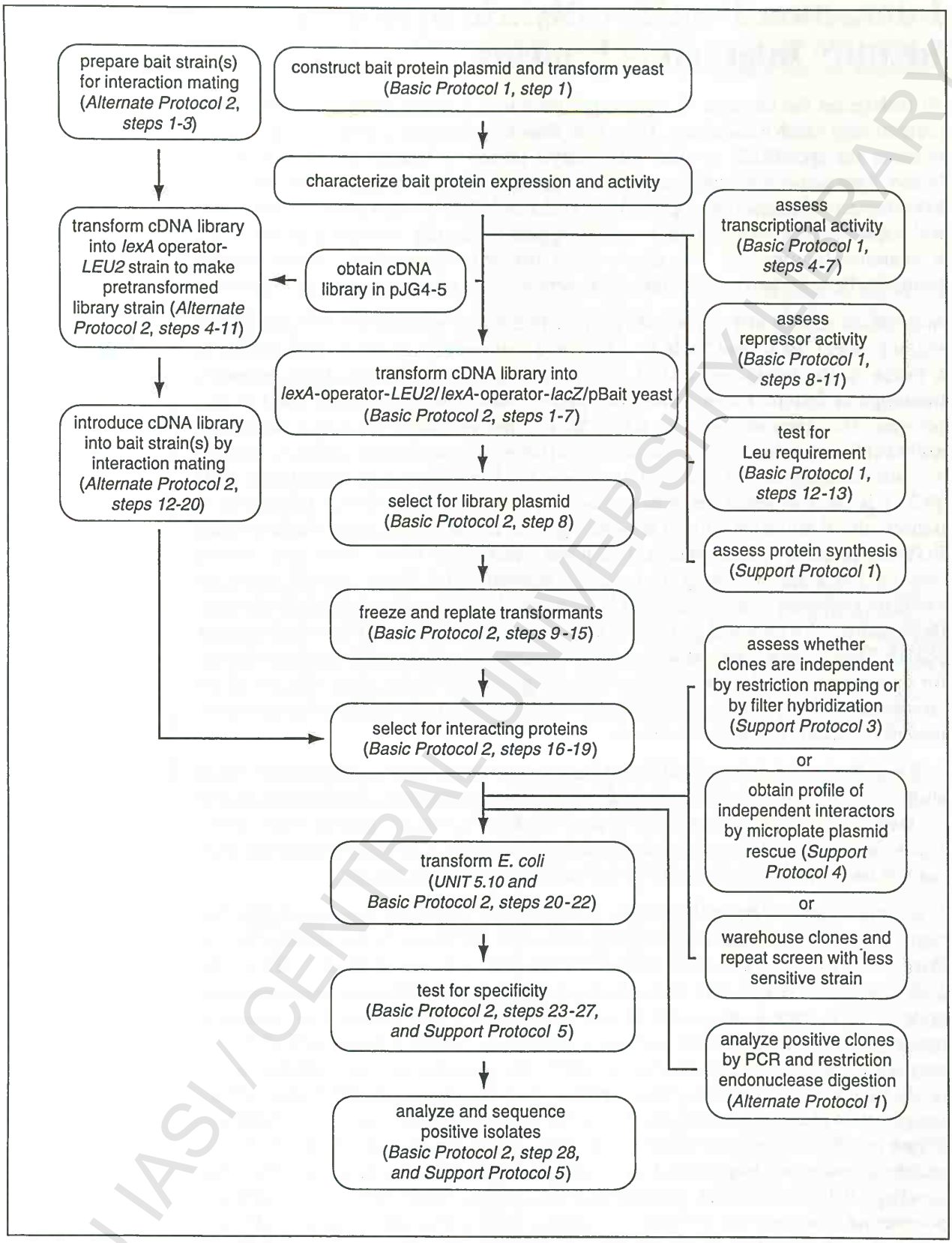

Figure 19.2.1 Flow chart for performing an interaction trap.

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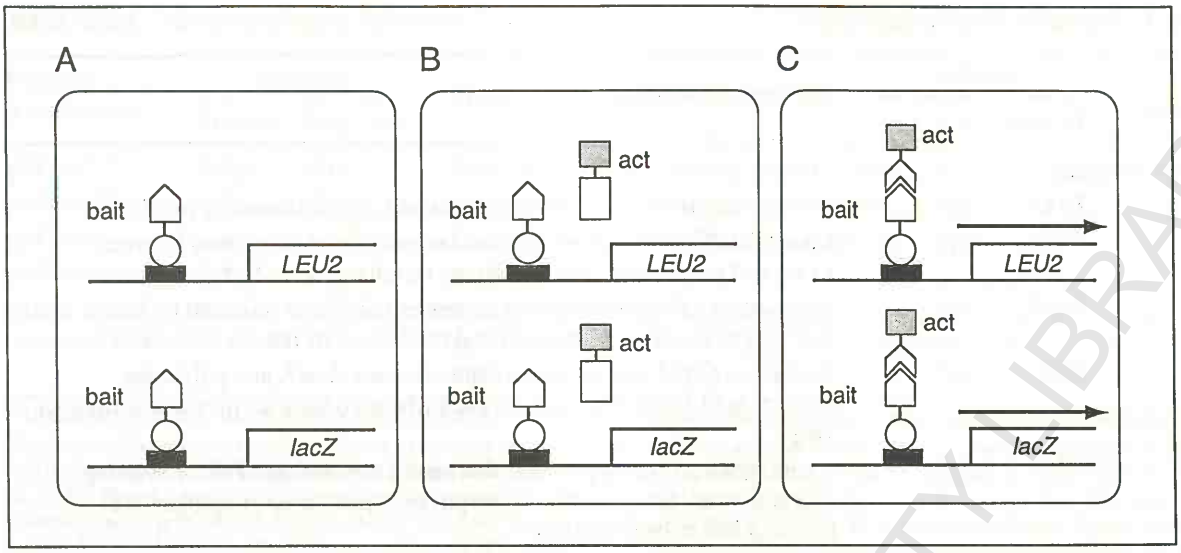

Figure 19.2.2 The interaction trap. (**A**) An EGY48 yeast cell containing two *LexA* operator–responsive reporters, one a chromosomally integrated copy of the *LEU2* gene (required for growth on –Leu medium), the second a plasmid bearing a *GAL1* promoter–*lacZ* fusion gene (causing yeast to turn blue on medium containing Xgal). The cell also contains a constitutively expressed chimeric protein, consisting of the DNA-binding domain of LexA fused to the probe or bait protein, shown as being unable to activate either of the two reporters. (**B**) and (**C**), EGY48/pSH18-34/pbait-containing yeast have been additionally transformed with an activation domain (act)–fused cDNA library in pJG4-5, and the library has been induced. In (B), the encoded protein does not interact specifically with the bait protein and the two reporters are not activated. In (C), a positive interaction is shown in which the library-encoded protein interacts with bait protein, resulting in activation of the two reporters (arrow), thus causing growth on medium lacking Leu and blue color on medium containing Xgal. Symbols: black rectangle, *LexA* operator sequence; open circle, LexA protein; open pentagon, bait protein; open rectangle, library protein; shaded box, activator protein (acid blob in Fig. 19.2.6).

When more than one bait will be used to screen a single library, significant time and resources can be saved by performing the interactor hunt by interaction mating (see Alternate Protocol 2). In this protocol, EGY48 is transformed with library DNA and the transformants are collected and frozen in aliquots. For each interactor hunt, an aliquot of the pretransformed EGY48/library strain is thawed and mixed with an aliquot of a bait strain transformed with the bait expression plasmid and pSH18-34. Overnight incubation of the mixture on a YPD plate results in fusion of the two strains to form diploids. The diploids are then exposed to galactose to induce expression of the library-encoded proteins, and interactors are selected in the same manner as in Basic Protocol 2. The advantage to this approach is that it requires only one high-efficiency library transformation for multiple hunts with different baits. It is also useful for bait proteins that are somewhat toxic to yeast; yeast expressing toxic baits can be difficult to transform with the library DNA.

CHARACTERIZING A BAIT PROTEIN

BASIC PROTOCOL 1

The first step in an interactor hunt is to construct a plasmid that expresses LexA fused to the protein of interest. This construct is transformed into reporter yeast strains containing *LEU2* and *lacZ* reporter genes, and a series of control experiments is performed to establish whether the construct is suitable as is or must be modified, and whether alternative yeast reporter conditions should be used. These controls establish that the bait protein is made as a stable protein in yeast, that it is capable of entering the nucleus and binding *LexA* operator sites, and that it does not appreciably activate transcription of the *LexA* operator–based reporter genes. This last is the most important constraint on use of this system. The LexA-fused bait protein must not activate transcription of either re-

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Table 19.2.1 Interaction Trap Components[a,b]

Plasmid name/source	Selection		Comment/description
	In yeast	In *E. coli*	
LexA fusion plasmids			
pEG202[c,d,e]	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses LexA followed by polylinker
pJK202	*HIS3*	Ap^r	Like pEG202, but incorporates nuclear localization sequences between LexA and polylinker; used to enhance translocation of bait to nucleus
pNLexA[e]	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses polylinker followed by LexA; for use with baits where amino-terminal residues must remain unblocked
pGilda[d]	*HIS3*	Ap^r	Contains a *GAL1* promoter that expresses same LexA and polylinker cassette as pEG202; for use with baits whose continuous presence is toxic to yeast
pEE202I	*HIS3*	Ap^r	An integrating form of pEG202 that can be targeted into *HIS3* following digestion with *Kpn*I; for use where physiological screen requires lower levels of bait to be expressed
pRFHM1[e,f] (control)	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses LexA fused to the homeodomain of bicoid to produce nonactivating fusion; used as positive control for repression assay, negative control for activation and interaction assays
pSH17-4[e,f] (control)	*HIS3*	Ap^r	*ADH* promoter expresses LexA fused to GAL4 activation domain; used as a positive control for transcriptional activation
pMW101[f]	*HIS3*	Cm^r	Same as pEG202, but with altered antibiotic resistance markers; basic plasmid used for cloning bait
pMW103[f]	*HIS3*	Km^r	Same as pEG202, but with altered antibiotic resistance markers; basic plasmid used for cloning bait
pHybLex/Zeo[f,g]	Zeo^r	Zeo^r	Bait cloning vector compatible with interaction trap and all other two-hybrid systems; minimal ADH promotor expresses LexA followed by extended polylinker
Activation domain fusion plasmids			
pJG4-5[c,d,e,f]	TRP1	Ap^r	Contains a *GAL1* promoter that expresses nuclear localization domain, transcriptional activation domain, HA epitope tag, cloning sites; used to express cDNA libraries
pJG4-5I	*TRP1*	Ap^r	An integrating form of pJG4-5 that can be targeted into *TRP1* by digestion with *Bsu*36I (New England Biolabs); to be used with pEE202I to study interactions that occur physiologically at low protein concentrations
pYESTrp[g]	*TRP1*	Ap^r	Contains a *GAL1* promoter that expresses nuclear localization domain, transcriptional activation domain, V5 epitope tag, multiple cloning sites; contains f1 ori and T7 promoter/flanking site; used to express cDNA libraries (Invitrogen)
pMW102[f]	*TRP1*	Km^r	Same as pJG4-5, but with altered antibiotic resistance markers; no libraries yet available
pMW104[f]	*TRP1*	Cm^r	Same as pJG4-5, but with altered antibiotic resistance markers; no libraries yet available
LacZ reporter plasmids			
pSH18-34[d,e,f]	*URA3*	Ap^r	Contains 8 *LexA* operators that direct transcription of the *lacZ* gene; one of the most sensitive indicator plasmids for transcriptional activation
pJK103[e]	*URA3*	Ap^r	Contains two *LexA* operators that direct transcription of the *lacZ* gene; an intermediate reporter plasmid for transcriptional activation
pRB1840[e]	*URA3*	Ap^r	Contains 1 *LexA* operator that directs transcription of the *lacZ* gene; one of the most stringent reporters for transcriptional activation
pMW112[f]	*URA3*	Km^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW109[f]	*URA3*	Km^r	Same as pJK103, but with altered antibiotic resistance marker

continued

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Table 19.2.1 Interaction Trap Components[a,b], continued

Plasmid name/source	Selection		Comment/description
	In yeast	In *E. coli*	
pMW111[f]	*URA3*	Km^r	Same as pRB1840, but with altered antibiotic resistance marker
pMW107[f]	*URA3*	Cm^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW108[f]	*URA3*	Cm^r	Same as pJK103, but with altered antibiotic resistance marker
pMW110[f]	*URA3*	Cm^r	Same as pRB1840, but with altered antibiotic resistance marker
pJK101[e,f] (control)	*URA3*	Ap^r	Contains a *GAL1* upstream activating sequence followed by two *lexA* operators followed by *lacZ* gene; used in repression assay to assess bait binding to operator sequences

[a]All plasmids contain a 2μm origin for maintenance in yeast, as well as a bacterial origin of replication, except where noted (pEE202I, pJG4.5I).

[b]Interaction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Plasmids with altered antibiotic resistance markers (all pMW plasmids) were constructed at Glaxo in Research Triangle Park, N.C. (Watson et al., 1996). Plasmids and strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (pEG202); J. Kamens, BASF, Worcester, Mass. (pJK202); cumulative efforts of I. York, Dana-Farber Cancer Center, Boston, Mass. and M. Sainz and S. Nottwehr, U. Oregon (pNLexA); D.A. Shaywitz, MIT Center for Cancer Research, Cambridge, Mass. (pGilda); R. Buckholz, Glaxo, Research Triangle Park, N.C. (pEE2021, pJG4-51); J. Gyuris, Mitotix, Cambridge, Mass. (pJG4-5); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH17-4); R.L. Finley, Wayne State University School of Medicine, Detroit, Mich. (pRFHM1); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH18-34); J. Kamens, BASF, Worcester, Mass. (pJK101, pJK103); R. Brent, Dept. of Molecular Biology, Massachusetts General Hospital, Boston, Mass. (pRB1840). Specialized plasmids not yet commercially available can be obtained by contacting the Brent laboratory at Massachusetts General Hospital, (617) 726-5925 or *brent@frodo.mgh.harvard.edu*, or the Golemis laboratory, (215) 728-2860 or *EA_Golemis@fccc.edu*.

[c]Sequence data are available for pEG202 (pLexA) accession number pending.

[d]Plasmids commercially available from Clontech and OriGene; for Clontech pEG202 is listed as pLexA, pJG4-5 as pB42AD, and pSH18-34 as p8op-LacZ.

[e]Plasmids and strains available from OriGene.

[f]In pMW plasmids the ampicillin resistance gene (Ap^r) is replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r) from pBC SK(+) and pBK-CMV (Stratagene), respectively. The choice between Km^r and Cm^r or Ap^r plasmids is a matter of personal taste; use of basic Ap^r plasmids is described in the basic protocols. Use of the more recently developed reagents would facilitate the purification of library plasmid in later steps by eliminating the need for passage through KC8 bacteria, with substantial saving of time and effort. Ap^r has been maintained as marker of choice for the library plasmid because of the existence of multiple libraries already possessing this marker. These plasmids are the basic set of plasmids recommended for use.

[g]Plasmids commercially available from Invitrogen as components of a Hybrid Hunter kit; this kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

porter—the EGY48 strain (or related strain EGY191) that expresses the LexA fusion protein should not grow on medium lacking Leu, and the colonies should be white on medium containing Xgal. The characterized bait protein plasmid is used for Basic Protocol 2 to screen a library for interacting proteins.

Materials

DNA encoding the protein of interest

Plasmids (see Table 19.2.1): pEG202 (see Fig. 19.2.3), pSH18-34 (see Fig. 19.2.4), pSH17-4, pRFHM1, and pJK101 for basic characterization; other plasmids for specific circumstances as described (Clontech, Invitrogen, OriGene, or R. Brent)

Yeast strain EGY48 (*ura3 trp1 his3 3LexA*-operator-*LEU2*), or EGY191 (*ura3 trp1 his3 1LexA*-operator-*LEU2*; Table 19.2.2)

Complete minimal (CM) medium dropout plates (*APPENDIX 4L*), supplemented with 2% (w/v) of the indicated sugars (glucose or galactose), in 100-mm plates:
- Glu/CM, –Ura, –His
- Gal/CM, –Ura, –His
- Gal/CM, –Ura, –His, –Leu

Z buffer (*APPENDIX 4L*) with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Xgal)

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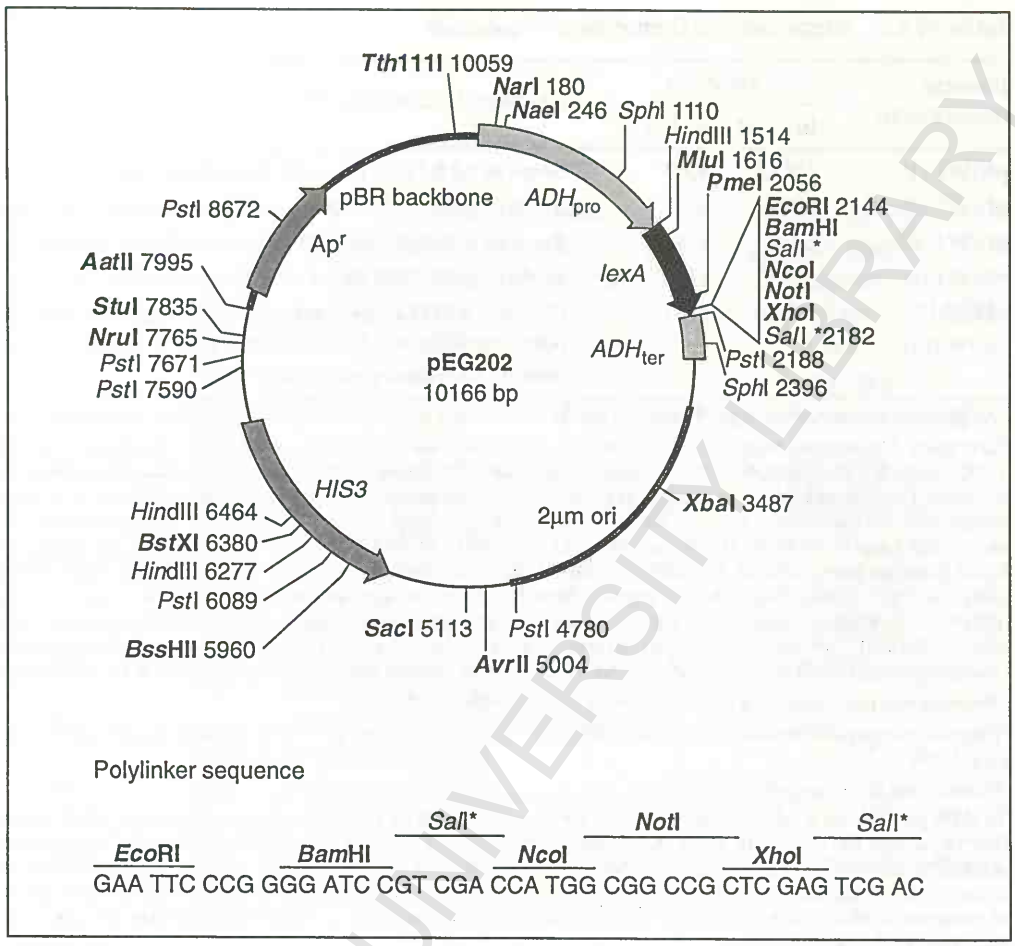

Figure 19.2.3 LexA-fusion plasmids: pEG202. The strong constitutive *ADH* promoter is used to express bait proteins as fusions to the DNA-binding protein LexA. Restriction sites shown in this map are based on recently compiled pEG202 sequence data and include selected sites suitable for diagnostic restriction endonuclease digests. A number of restriction sites are available for insertion of coding sequences to produce protein fusions with LexA; the polylinker sequence and reading frame relative to LexA are shown below the map with unique sites marked in bold type. The sequence 5′-CGT CAG CAG AGC TTC ACC ATT G-3′ can be used to design a primer to confirm correct reading frame for LexA fusions. Plasmids contain the *HIS3* selectable marker and the 2µm origin of replication to allow propagation in yeast, and the Ap^r antibiotic resistance gene and the pBR origin of replication to allow propagation in *E. coli*. In the recently developed LexA-expression plasmids pMW101 and pMW103, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r), respectively (see Table 19.2.1 for details).

Gal/CM dropout liquid medium (*APPENDIX 4L*) supplemented with 2% Gal

Antibody to LexA or fusion domain: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

H_2O, sterile

30°C incubator

Nylon membrane

Whatman 3MM filter paper

Additional reagents and equipment for subcloning DNA fragments (Struhl, 1987), lithium acetate transformation of yeast (*APPENDIX 4L*), liquid assay for β-galactosidase (*APPENDIX 4L*), preparation of protein extracts for immunoblot analysis (see Support Protocol 1), and immunoblotting and immunodetection (*UNIT 10.10*)

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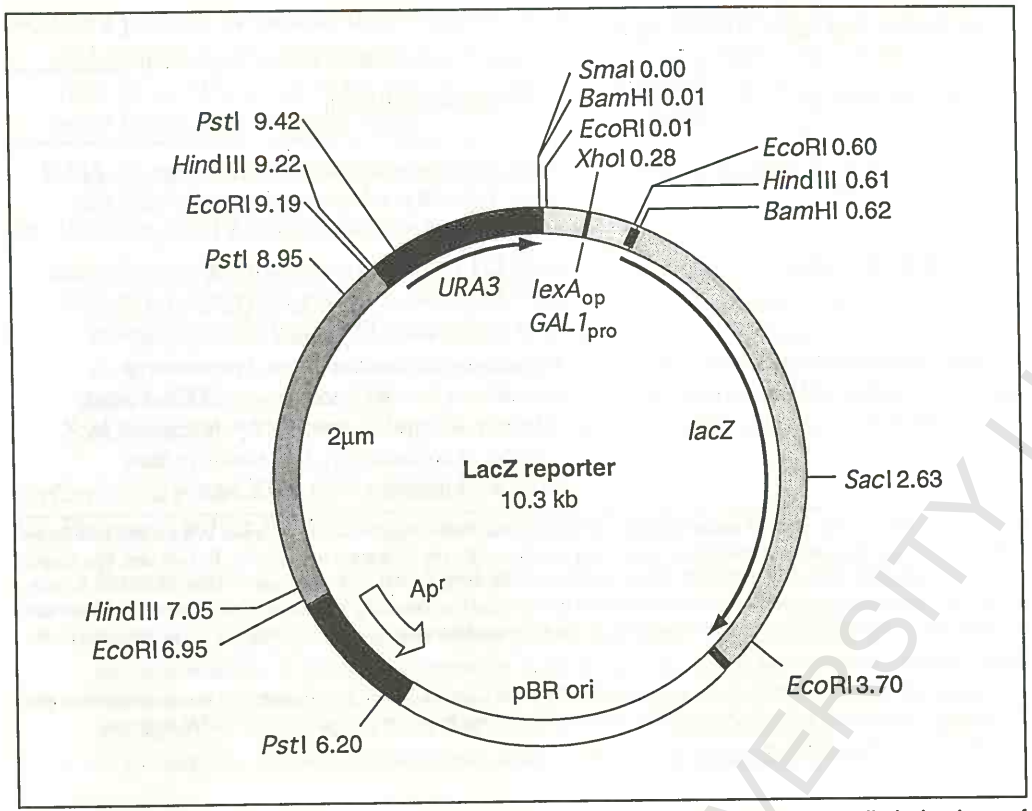

Figure 19.2.4 LacZ reporter plasmid. pRB1840, pJK103, and pSH18-34 are all derivatives of LR1Δ1 (West et al., 1984) containing eight, two, or one operator for LexA (*LexA*$_{op}$) binding inserted into the unique *Xho*I site located in the minimal *GAL1* promoter (*GAL1*$_{pro}$; 0.28 on map). The plasmid contains the *URA3* selectable marker, the 2µm origin to allow propagation in yeast, the ampicillin resistance (Ap^r) gene, and the pBR322 origin (ori) to allow propagation in *E. coli*. Numbers indicate relative map positions. In the recently developed derivatives, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol or kanamycin resistance genes (see Table 19.2.1 for details).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

Transform yeast with the bait protein plasmid

1. Using standard subcloning techniques (e.g., Struhl, 1987), insert the DNA encoding the protein of interest into the polylinker of pEG202 (see Fig. 19.2.3) or other LexA fusion plasmid to make an in-frame protein fusion.

   The LexA fusion protein is expressed from the strong alcohol dehydrogenase (ADH) promoter. pEG202 also contains a HIS3 selectable marker and a 2µm origin for propagation in yeast. pEG202 with the DNA encoding the protein of interest inserted is designated pBait. Uses of alternative LexA fusion plasmids are described in Background Information.

2. Perform three separate lithium acetate transformations (*APPENDIX 4L*) of EGY48 using the following combinations of plasmids:

   pBait + pSH18-34 (test)
   pSH17-4 + pSH18-34 (positive control for activation)
   pRFHM1 + pSH18-34 (negative control for activation).

   Use of the two LexA fusions as positive and negative controls allows a rough assessment of the transcriptional activation profile of LexA bait proteins. pEG202 itself is not a good negative control because the peptide encoded by the uninterrupted polylinker sequences is itself capable of very weakly activating transcription.

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Table 19.2.2 Interaction Trap Yeast Selection Strains[a]

Strain	Relevant genotype	Number of operators	Comments/description
EGY48[b,c,d]	*MATα trp1*, *his3*, *ura3*, lexAops-LEU2	6	*lexA* operators direct transcription from the *LEU2* gene; EGY48 is a basic strain used to select for interacting clones from a cDNA library
EGY191	*MATα trp1*, *his3*, *ura3*, lexAops-LEU2	2	EGY191 provides a more stringent selection than EGY48, producing lower background with baits with instrinsic ability to activate transcription
L40[c]	MATα *trpl*, *leu2*, *ade2*, GAL4, *lexAops-HIS34*, *lexAops-lacZ8*		Expression driven from *GAL1* promoter is constitutive in L40 (inducible in EGY strains); selection is for *HIS* prototrophy. Integrated *lacZ* reporter is considerably less sensitive than pSH18-34 maintained in EGY strains

[a]Interaction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (EGY48, EGY191); A.B. Vojtek and S.M. Hollenberg, Fred Hutchinson Cancer Research Center, Seattle, Wash. (L40). Specialized strains not yet commercially available can be obtained by contacting the Brent laboratory at The Molecular Sciences Institute, Berkeley, (510) 726-5990 or brent@molsci.org, or the Golemis laboratory, (215) 728-2860 or *EA_Golemis@fccc.edu.*

[b]Strains commercially available from Clontech.

[c]Strains commercially available from Invitrogen as components of a Hybrid Hunter kit; the kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

[d]Strains commercially available from OriGene.

pSH18-34 contains a 2μm origin and a URA3 selectable marker for maintenance in yeast, as well as a bacterial origin of replication and ampicillin-resistance gene. It is the most sensitive lacZ reporter available and will detect any potential ability to activate lacZ transcription. pSH17-4 is a HIS3 2μm plasmid encoding LexA fused to the activation domain of the yeast activator protein GAL4. This fusion protein strongly activates transcription. pRFHM1 is a HIS3 2μm plasmid encoding LexA fused to the N-terminus of the Drosophila protein bicoid. This fusion protein has no ability to activate transcription.

3. Plate each transformation mixture on Glu/CM –Ura, –His dropout plates. Incubate 2 days at 30°C to select for yeast that contain both plasmids.

   Colonies obtained can be used simultaneously in tests for the activation of lacZ (steps 4 to 7) and LEU2 (steps 12 to 13) reporters.

Assay lacZ gene activation by β-galactosidase assay

4. Streak a Glu/CM –Ura, –His master dropout plate with at least five or six independent colonies obtained from each of the three transformations in step 3 (test, positive control, and negative control) and incubate overnight at 30°C.

   The filter assay described in Steps 5a to 7a (based on Breeden and Nasmyth, 1985) provides a rapid assay for β-galactosidase transcription. Alternatively, a liquid assay (APPENDIX 4L) or a plate assay (described in Steps 5b to 7b) may be used.

Perform filter assay for β-galactosidase activity:

5a. Lift colonies by gently placing a nylon membrane on the yeast plate and allowing it to become wet through. Remove the membrane and air dry 5 min. Chill the membrane, colony side up, 10 min at –70°C.

   Whatman 3MM filters can be cut to the size of the yeast plate as a more economical alternative to nylon membranes for performing lifts. In addition, two or three 5-min temperature cycles (–70°C to room temperature) can be used instead of a single cycle to promote better lysis; this may be worth doing if there is difficulty visualizing blue color.

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6a. Cut a piece of Whatman 3MM filter paper slightly larger than the colony membrane and soak it in Z buffer containing 1 mg/ml Xgal. Place colony membrane, colony side up, on Whatman 3MM paper, or float it in the lid of a petri dish containing ~2 ml Z buffer with 1 mg/ml Xgal.

Acceptable results may be obtained using as little as 300 µg/ml Xgal.

7a. Incubate at 30°C and monitor for color changes.

It is generally useful to check the membrane after 20 min, and again after 2 to 3 hr. Strong activators will produce a blue color in 5 to 10 min, and a bait protein (LexA fusion protein) that does so is unsuitable for use in an interactor hunt using this lacZ reporter plasmid. Weak activators will produce a blue color in 1 to 6 hr (compare versus negative control pRFHMI which will itself produce a faint blue color with time) and may or may not be suitable. Weak activators should be tested using the repressor assay described in steps 8 to 11.

Perform Xgal plate assay for lacZ activation:

5b. Prepare Z buffer Xgal plates as described in APPENDIX 4L.

For activation assays, plates should be prepared with glucose as a sugar source. For repression assays (steps 8 to 11), galactose should be used as a sugar source. In our experience, when patching from a master plate to Xgal plates, sufficient yeast are transferred that plasmid loss is not a major problem even in the absence of selection; this is balanced by the desire to assay sets of constructs on the same plate to eliminate batch variation in Xgal potency. Hence, plates should be made either with complete minimal amino acid mix, or by dropping out only uracil (–Ura), to make the plates universally useful.

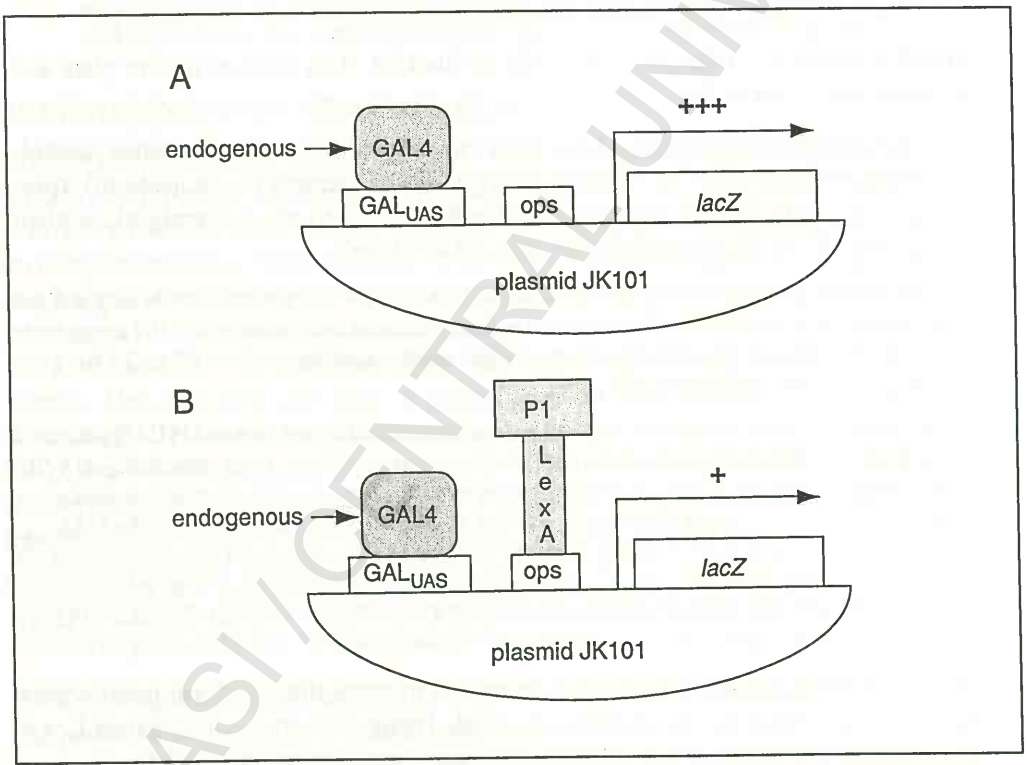

Figure 19.2.5 Repression assay for DNA binding. **(A)** The plasmid JK101 contains the upstream activating sequence (UAS) from the *GAL1* gene followed by *LexA* operators upstream of the *lacZ* coding sequence. Thus, yeast containing pJK101 will have significant β-galactosidase activity when grown on medium in which galactose is the sole carbon source because of binding of endogenous yeast GAL4 to the GAL_{UAS} **(B)**. LexA-fused proteins (P1-LexA) that are made, enter the nucleus, and bind the *LexA* operator sequences (ops) will block activation from the GAL_{UAS}, repressing β-galactosidase activity (+) 3- to 5-fold. On glucose/Xgal medium, yeast containing pJK101 should be white because GAL_{UAS} transcription is repressed.

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6b. Streak yeast from master plate to Xgal plate and incubate at 30°C.

7b. Examine plates for color development at intervals over the next 2 to 3 days.

Strongly activating fusions should be visibly blue on the plate within 12 to 24 hr; moderate activators will be visibly blue after ~2 days.

When a bait protein appreciably activates transcription under these conditions, there are several recourses. The first and simplest is to switch to a less sensitive lacZ reporter plasmid; use of pJK103 and pRB1840 may be sufficient to reduce background to manageable levels. If this fails to work, it is frequently possible to generate a truncated LexA fusion that does not activate transcription.

Confirm fusion-protein synthesis by repression assay

For LexA fusions that do not activate transcription, confirm by performing a repression assay (Brent and Ptashne, 1984) that the LexA fusion protein is being synthesized in yeast (some proteins are not) and that it is capable of binding *LexA* operator sequences (Fig. 19.2.5). The following steps can be performed concurrently with the activation assay.

8. Transform EGY48 yeast with the following combinations of plasmids (three transformations):

   pBait + pJK101 (test)
   pRFHM1 + pJK101 (positive control for repression)
   pJK101 alone (negative control for repression).

9. Plate each transformation mix on Glu/CM –Ura, –His dropout plates or Glu/CM –Ura dropout plates as appropriate to select yeast cells that contain the indicated plasmids. Incubate 2 to 3 days at 30°C until colonies appear.

10. Streak colonies to a Glu/CM –Ura, –His or Glu/CM –Ura dropout master plate and incubate overnight at 30°C.

11. Assay β-galactosidase activity of the three transformed strains (test, positive control, and negative control) by liquid assay (using Gal/CM dropout liquid medium), filter assay (steps 5a to 7a, first restreaking to Gal/CM plates to grow overnight), or plate assay (steps 5b to 7b, using Gal/CM –Ura XGal plates).

    This assay should not be run for more than 1 to 2 hr for membranes, or 36 hr for Xgal plates, as the high basal lacZ activity will make differential activation of pJK101 impossible to see with longer incubations. Use of Xgal plates, and inspection 12 to 24 hr after streaking, is generally most effective.

    The plasmid pJK101 contains the galactose upstream activating sequence (UAS) followed by LexA operators upstream of the lacZ coding sequence. Thus, yeast containing pJK101 will have significant β-galactosidase activity when grown on medium in which galactose is the sole carbon source because of binding of endogenous yeast GAL4 to the GAL_{UAS}. LexA-fused proteins that are made, enter the nucleus, and bind the LexA operator sequences block activation from the GAL_{UAS}, repressing β-galactosidase activity 3- to 20-fold. Note that on Glu/Xgal medium, yeast containing pJK101 should be white, because GAL_{UAS} transcription is repressed.

12. If a bait protein neither activates nor represses transcription, perform immunoblot analysis by probing an immunoblot of a crude lysate with antibodies against LexA or the fusion domain to test for protein synthesis (see Support Protocol 1).

    Even if a bait protein represses transcription, it is generally a good idea to assay for the production of full-length LexA fusions, as occasionally some fusion proteins will be proteolytically cleaved by endogenous yeast proteases. If the protein is made but does not repress, it may be necessary to clone the sequence into a LexA fusion vector that contains a nuclear localization motif, e.g., pJK202 (see Table 19.2.1), or to modify or truncate the fusion domain to remove motifs that target it to other cellular compartments (e.g., myristoylation signals).

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Test for Leu requirement

These steps can be performed concurrently with the *lacZ* activation and repression assays.

13. Disperse a colony of EGY48 containing pBait and pSH18-34 reporter plasmids into 500 µl sterile water. Dilute 100 µl of suspension into 1 ml sterile water. Make a series of 1/10 dilutions in sterile water to cover a 1000-fold concentration range.

14. Plate 100 µl from each tube (undiluted, 1/10, 1/100, and 1/1000) on Gal/CM –Ura, –His dropout plates and on Gal/CM –Ura, –His, –Leu dropout plates. Incubate overnight at 30°C.

There will be a total of eight plates. Gal/CM –Ura, –His dropout plates should show a concentration range from 10 to 10,000 colonies and Gal/CM –Ura, –His, –Leu dropout plates should have no colonies.

Actual selection in the interactor hunt is based on the ability of the bait protein and acid-fusion pair, but not the bait protein alone, to activate transcription of the LexA operator-LEU2 gene and allow growth on medium lacking Leu. Thus, the test for the Leu requirement is the most important test of whether the bait protein is likely to have an unworkably high background. The LEU2 reporter in EGY48 is more sensitive than the pSH18-34 reporter for some baits, so it is possible that a bait protein that gives little or no signal in a β-galactosidase assay would nevertheless permit some level of growth on –Leu medium. If this occurs, there are several options for proceeding, the most immediate of which is to substitute EGY191 (see Table 19.2.2), a less sensitive screening strain, and repeat the assay.

As outlined in this protocol, the authors recommend the strategy of performing the initial screening using the most sensitive reporters and then, if activation is detected, screening with increasingly less sensitive reporters (see Critical Parameters for further discussion).

PERFORMING AN INTERACTOR HUNT

BASIC PROTOCOL 2

An interactor hunt involves two successive large platings of yeast containing LexA-fused probes and reporters and libraries in pJG4-5 (Fig. 19.2.6, Table 19.2.3) with a cDNA expression cassette under control of the *GAL* promoter. In the first plating, yeast are plated on complete minimal (CM) medium –Ura, –His, –Trp dropout plates with glucose (Glu) as a sugar source to select for the library plasmid. In the second plating, which selects for yeast that contain interacting proteins, a slurry of primary transformants is plated on CM –Ura, –His, –Trp, –Leu dropout plates with galactose/raffinose (Gal/Raff) as the sugar source. This two-step selection is encouraged for two reasons. First, a number of interesting cDNA-encoded proteins may be deleterious to the growth of yeast that bear them; these would be competed out in an initial mass plating. Second, it seems likely that immediately after simultaneous transformation and Gal induction, yeast bearing particular interacting proteins may not be able to initially express sufficient levels of these proteins to support growth on medium lacking Leu. Library plasmids from colonies identified in the second plating are purified by bacterial transformation and used to transform yeast cells for the final specificity screen.

A list of libraries currently available for use with this system is provided in Table 19.2.3. The protocol outlined below describes the steps used to perform a single-step screen that should saturate a library derived from a mammalian cell. For screens with libraries derived from lower eukaryotes with less complex genomes, fewer plates will be required.

Occasionally, baits that seemed well-behaved during preliminary tests produce unworkably high backgrounds of "positives" during an actual screen (see Background Information and Critical Parameters). To forestall the waste of time and materials performing a screen with such a bait would entail, an alternative approach is to perform a scaled-back

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screen when working with a new bait (e.g., 5 rather than 30 plates of primary transformants). The results can be assessed before doing a full screen; it is then possible to switch to lower-sensitivity reporter strains and plasmids, if appropriate. Although individual baits will vary, the authors' current default preference is to use the *lacZ* reporter pJK103 in conjunction with either EGY48 or EGY191. Polymerase chain reaction (PCR) can also be used in a rapid screening approach that may be preferable if a large number of positions are obtained in a library screen (see Alternate Protocol 1).

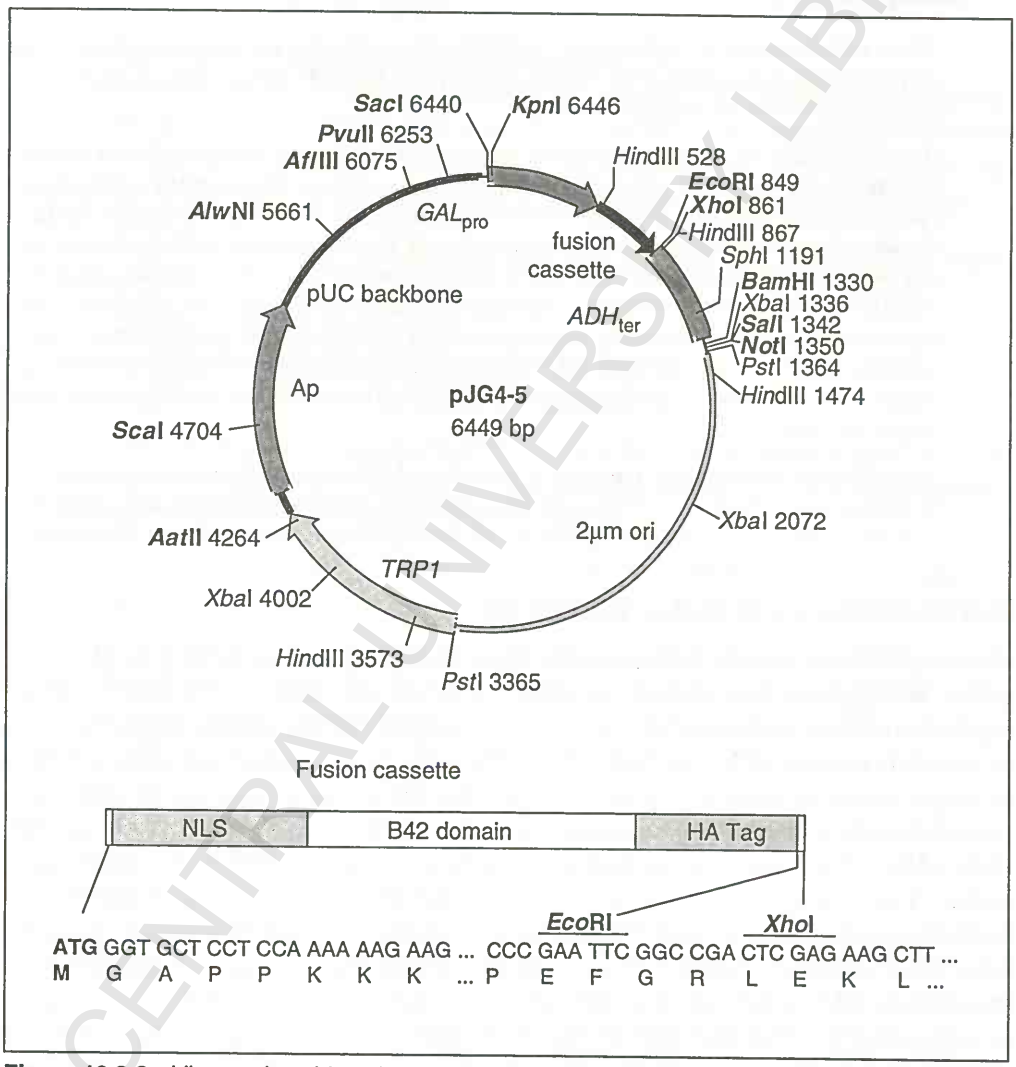

Figure 19.2.6 Library plasmids: pJG4-5. Library plasmids express cDNAs or other coding sequences inserted into unique *Eco*RI and *Xho*I sites as a translational fusion to a cassette consisting of the SV40 nuclear localization sequence (NLS; PPKKKRKVA), the acid blob B42 domain (Ruden et al, 1991), and the hemagglutinin (HA) epitope tag (YPYDVPDYA). Expression of cassette sequences is under the control of the *GAL1* galactose-inducible promoter. This map is based on the sequence data available for pJG4-5, and includes selected sites suitable for diagnostic restriction digests (shown in bold). The sequence 5′-CTG AGT GGA GAT GCC TCC-3′ can be used as a primer to identify inserts or to confirm correct reading frame. The pJG4-5 plasmid contains the *TRP1* selectable marker and the 2µm origin to allow propagation in yeast, and the antibiotic resistance gene and the pUC origin to allow propagation in *E. coli.* In the recently developed pJG4-5 derivative plasmids pMW104 and pMW102, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r), respectively (see Table 19.2.2 for details). Currently existing libraries are all made in the pJG4-5 plasmid (Gyuris et al., 1993) shown on this figure. Unique sites are marked in bold type.

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Materials

Yeast containing appropriate combinations of plasmids (see Table 19.2.1 and Table 19.2.2):
- EGY48 containing *LexA*-operator-*lacZ* reporter and pBait (see Basic Protocol 1)
- EGY48 containing *LexA*-operator-*lacZ* reporter and pRFHM-1
- EGY48 containing *LexA*-operator-*lacZ* reporter and any nonspecific bait

Complete minimal (CM) dropout liquid medium (*APPENDIX 4L*) supplemented with sugars (glucose, galactose, and/or raffinose) as indicated [2% (w/v) Glu, or 2% (w/v) Gal + 1% (w/v) Raff]:
- Glu/CM –Ura, –His
- Glu/CM –Trp
- Gal/Raff/CM –Ura, –His, –Trp

H_2O, sterile

TE buffer (pH 7.5; *APPENDIX 2E*)/0.1 M lithium acetate

Library DNA in pJG4-5 (Table 19.2.3 and Fig. 19.2.6)

High-quality sheared salmon sperm DNA (see Support Protocol 2)

40% (w/v) polyethylene glycol 4000 (PEG 4000; filter sterilized)/0.1 M lithium acetate/TE buffer (pH 7.5)

Dimethyl sulfoxide (DMSO)

Complete minimal (CM) medium dropout plates (*APPENDIX 4L*) supplemented with sugars and Xgal (20 µg/ml) as indicated [2% (w/v) Glu, and 2% (w/v) Gal + 1% (w/v) Raff]:
- Glu/CM –Ura, –His, –Trp, 24 × 24–cm (Nunc) and 100-mm
- Gal/Raff/CM –Ura, –His, –Trp, 100-mm
- Gal/Raff/CM –Ura, –His, –Trp, –Leu, 100-mm
- Glu/Xgal/CM –Ura, –His, –Trp, 100-mm
- Gal/Raff/Xgal/CM –Ura, –His, –Trp, 100-mm
- Glu/CM –Ura, –His, –Trp, –Leu, 100-mm
- Glu/CM –Ura, –His, 100-mm
- Gal/CM –Ura, –His, –Trp, –Leu, 100-mm

TE buffer (pH 7.5), sterile (optional)

Glycerol solution (see recipe)

E. coli KC8 (*pyrF leuB600 trpC hisB463*; constructed by K. Struhl and available from R. Brent)

LB/ampicillin plates (*APPENDIX 4A*)

E. coli DH5α or other strain suitable for preparation of DNA for sequencing

Bacterial defined minimal A medium plates: 1× A medium plates containing 0.5 µg/ml vitamin B1 (*APPENDIX 4A*) and supplemented with 40 µg/ml each Ura, His, and Leu

30°C incubator, with and without shaking

Low-speed centrifuge and rotor

50-ml conical tubes, sterile

1.5-ml microcentrifuge tubes, sterile

42°C heating block

Glass microscope slides, sterile

Additional reagents and equipment for rapid miniprep isolation of yeast DNA (*APPENDIX 4L*), transformation of bacteria by electroporation (*UNIT 5.10*), miniprep isolation of bacterial DNA (*APPENDIX 4C*), restriction endonuclease digestion (*APPENDIX 4I*; optional), and agarose gel electrophoresis (*APPENDIX 4F*; optional)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

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Table 19.2.3 Libraries Compatible with the Interaction Trap System[a]

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[b]	Contact information
Cell lines				
HeLa cells (human cervical carcinoma)	JG	9.6×10^6	0.3-3.5 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
HeLa cells (human cervical carcinoma)	Y	3.7×10^6	0.3-1.2 kb	Invitrogen
WI-38 cells (human lung fibroblasts), serum-starved, cDNA	JG	5.7×10^6	0.3-3.5 kb (1.5 kb)	R. Brent, Clontech, OriGene
Jurkat cells (human T cell leukemia), exponentially growing, cDNA	JG	4.0×10^6	0.7-2.8 kb (1.5 kb)	R. Brent
Jurkat cells (human T cell leukemia)	Y	3.2×10^6	0.3-1.2 kb	Invitrogen
Jurkat cells (human T cell leukemia)	Y	3.0×10^6	0.5-4.0 kb (1.8 kb)	Clontech
Jurkat cells (human T cell leukemia)	JG	5.7×10^6	(>1.3)	OriGene
Jurkat cells (human T cell leukemia)	JG	2×10^6	0.7-3.5 kb (1.2 kb)	S. Witte
Be Wo cells (human fetal placental choriocarcinoma)	Y	5.4×10^6	0.3-0.8 kb	Invitrogen
Human lymphocyte	JG	4.0×10^6	0.4-4.0 kb (2.0 kb)	Clontech
$CD4^+$ T cell, murine, cDNA	JG	$>10^6$	0.3-2.5 kb (>0.5 kb)	R. Brent
Chinese hamster ovary (CHO) cells, exponentially growing, cDNA	JG	1.5×10^6	0.3-3.5 kb	R. Brent
A20 cells (mouse B cell lymphoma)	Y	3.11×10^6	0.3-1.2 kb	Invitrogen
Human B cell lymphoma	JG	—	—	H. Niu
Human 293 adenovirus–infected (early and late stages)	JG	—	—	K. Gustin
SKOV3 human Y ovarian cancer	Y	5.0×10^6	(>1.4 kb)	OriGene
MDBK cell, bovine kidney	JG	5.8×10^6	(>1.2 kb)	OriGene
MDCK cells	JG	—	—	D. Chen
HepG2 cell line cDNA	JG	2×10^6	—	M. Melegari
MCF7 breast cancer cells, untreated	JG	1.0×10^7	(>1.5 kb)	OriGene
MCF7 breast cancer cells, estrogen-treated	JG	1.0×10^7	(>1.1 kb)	OriGene
MCF7 cells, serum-grown	JG	1.0×10^7	0.4-3.5 kb	OriGene
LNCAP prostate cell line, untreated	JG	2.9×10^6	(>0.8 kb)	OriGene
LNCAP prostate cell line, androgen-treated	JG	4.6×10^6	(>0.9 kb)	OriGene
Mouse pachytene spermatocytes	JG	—	—	C. Hoog
Tissues				
Human breast	Y	9×10^6	0.4-1.2 kb	Invitrogen
Human breast tumor	Y	8.84×10^6	0.4-1.2 kb	Invitrogen
Human liver	JG	$>10^6$	0.6-4.0 kb (>1 kb)	R. Brent
Human liver	Y	2.2×10^6	0.5-4 kb (1.3 kb)	Clontech
Human liver	JG	3.2×10^6	0.3-1.2 kb	Invitrogen
Human liver	JG	1.1×10^7	(> 1 kb)	OriGene
Human lung	Y	5.9×10^6	0.4-1.2 kb	Invitrogen
Human lung tumor	Y	1.9×10^6	0.4-1.2	Invitrogen
Human brain	JG	3.5×10^6	0.5-4.5 kb (1.4 kb)	Clontech
Human brain	Y	8.9×10^6	0.3-1.2 kb	Invitrogen
Human testis	Y	6.4×10^6	0.3-1.2 kb	Invitrogen
Human testis	JG	3.5×10^6	0.4-4.5 kb (1.6 kb)	Clontech
Human ovary	Y	4.6×10^6	0.3-1.2 kb	Invitrogen

continued

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Table 19.2.3 Libraries Compatible with the Interaction Trap System[a], continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[b]	Contact information
Human ovary	JG	4.6×10^6	(>1.3 kb)	OriGene
Human ovary	JG	3.5×10^6	0.5-4.0 kb (1.8 kb)	Clontech
Human heart	JG	3.0×10^6	0.3-3.5 kb (1.3 kb)	Clontech
Human placenta	Y	4.8×10^6	0.3-1.2 kb	Invitrogen
Human placenta	JG	3.5×10^6	0.3-4.0 kb (1.2 kb)	Clontech
Human mammary gland	JG	3.5×10^6	0.5-5 kb (1.6 kb)	Clontech
Human peripheral blood leucocyte	JG	1.0×10^7	(>1.3 kb)	OriGene
Human kidney	JG	3.5×10^6	0.4-4.5 kb (1.6 kb)	Clontech
Human fetal kidney	JG	3.0×10^6	(>1 kb)	OriGene
Human spleen	Y	1.14×10^7	0.4-1.2 kb	Invitrogen
Human prostate	Y	5.5×10^6	0.4-1.2 kb	Invitrogen
Human normal prostate	JG	1.4×10^6	0.4-4.5 kb (1.7 kb)	Clontech
Human prostate	JG	1.4×10^6	(>1 kb)	OriGene
Human prostate cancer	JG	1.1×10^6	(>0.9 kb)	OriGene
Human fetal prostate	JG	—	—	OriGene
Human fetal liver	JG	3.5×10^6	0.3-4.5 kb (1.3 kb)	Clontech
Human fetal liver	Y	2.37×10^6	0.3-1.2 kb	Invitrogen
Human fetal liver	JG	8.6×10^6	(>1 kb)	OriGene
Human fetal brain	JG	3.5×10^6	0.5-1.2 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
Mouse brain	JG	6.1×10^6	(>1 kb)	OriGene
Mouse brain	JG	4.5×10^6	0.4-4.5 kb (1.2 kb)	Clontech
Mouse breast, lactating	JG	1.0×10^7	0.4-3.1 kb	OriGene
Mouse breast, involuting	JG	1.0×10^7	0.4-7.0 kb	OriGene
Mouse breast, virgin	JG	1.0×10^7	0.4-5.5 kb	OriGene
Mouse breast, 12 days pregnant	JG	6.3×10^6	0.4-5.3 kb	OriGene
Mouse skeletal muscle	JG	7.2×10^6	0.4-3.5 kb	OriGene
Rat adipocyte, 9-week-old Zucker rat	JG	1.0×10^7	0.4-5.0 kb	OriGene
Rat brain	JG	4.5×10^6	0.3-3.4 kb	OriGene
Rat brain (day 18)	JG	—	—	H. Niu
Rat testis	JG	8.0×10^6	(>1.2 kb)	OriGene
Rat thymus	JG	8.2×10^6	(>1.3 kb)	OriGene
Mouse liver	JG	9.5×10^6	(>1.4 kb)	OriGene
Mouse spleen	JG	1.0×10^7	(>1 kb)	OriGene
Mouse ovary	JG	4.0×10^6	(>1.2 kb)	OriGene
Mouse prostate	JG	—	—	OriGene
Mouse embryo, whole (19-day)	JG	1.0×10^5	0.2-2.5 kb	OriGene
Mouse embryo	JG	3.6×10^6	0.5-5 kb (1.7 kb)	Clontech
Drosophila melanogaster, adult, cDNA	JG	1.8×10^6	(>1.0 kb)	OriGene
D. melanogaster, embryo, cDNA	JG	3.0×10^6	0.5-3.0 kb (1.4 kb)	Clontech
D. melanogaster, 0-12 hr embryos, cDNA	JG	4.2×10^6	0.5-2.5 kb (1.0 kb)	R. Brent
D. melanogaster, ovary, cDNA	JG	3.2×10^6	0.3-1.5 kb (800 bp)	R. Brent
D. melanogaster, disc, cDNA	JG	4.0×10^6	0.3-2.1 kb (900 bp)	R. Brent
D. melanogaster, head	JG	—	—	M. Rosbash

continued

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Table 19.2.3 Libraries Compatible with the Interaction Trap System[a], continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[b]	Contact information
Miscellaneous				
Synthetic aptamers	PJM-1	$>1\times 10^9$	60 bp	R. Brent
Saccharomyces cerevisiae, S288C, genomic	JG	$>3\times 10^6$	0.8-4.0 kb	R. Brent
S. cerevisiae, S288C, genomic	JG	4.0×10^6	0.5-4.0 kb	OriGene
Sea urchin ovary	JG	3.5×10^6	(1.7 kb)	Clontech
Caenorhabditis elegans	JG	3.8×10^6	(>1.2 kb)	OriGene
Agrobacterium tumefaciens	JG	—	—	—
Arabidopsis thaliana, 7-day-old seedlings	JG	—	—	H.M. Goodman
Tomato (*Lycopersicon esculentum*)	JG	8×10^6	—	G.B. Martin
Xenopus laevis embryo	JG	2.2×10^6	0.3-4 kb (1.0 kb)	Clontech

[a]Most libraries are constructed in either the pJG4-5 vector or the pYESTrp vector (JG or Y in the Vector column); the peptide aptamer library is made in the pJM-1 vector. Libraries available from the public domain were constructed by the following individuals: (1) J. Gyuris; (3) C. Sardet and J. Gyuris; (4) W. Kolanus, J. Gyuris, and B. Seed; (39) D. Krainc; (50-52) R. Finley; (55) P. Watt; (54) P. Colas, B. Cohen, T. Jessen, I. Grishina, J. McCoy, and R. Brent (Colas et al., 1996). All libraries mentioned above were constructed in conjunction with and are available from the laboratory of Roger Brent, (617) 726-5925 or *brent@frodo.mgh.harvard.edu*. The following individual investigators must be contacted directly: (18) J. Pugh, Fox Chase Cancer Center, Philadelphia, Pa.; (8,9) Vinyaka Prasad, Albert Einstein Medical Center New York, N.Y.; (57, 58) Gregory B. Martin, *gmartin@dept.agry.purdue.edu*; (11) Huifeng Niu, *hn34@columbia.edu*; (16) Christer Hoog, *christer.hoog@cmb.ki.se*; (12) Kurt Gustin, *kgus@umich.edu*; (6) Stephan Witte, *Stephan.Witte@nimbus.rz.uni-konstanz.de*.

[b]Insert size ranges for pJG4-5 based libraries originally constructed in the Brent laboratory, which are now commercially available from Clontech, were reestimated by the company.

Transform the library

1. Grow an ~20-ml culture of EGY48 or EGY191 containing a *LexA*-operator-*lacZ* reporter plasmid and pBait in Glu/CM –Ura, –His liquid dropout medium overnight at 30°C.

   For best results, the pBait and lacZ reporter plasmids should have been transformed into the yeast within ~7 to 10 days of commencing a screen.

2. In the morning, dilute culture into 300 ml Glu/CM –Ura, –His liquid dropout medium to 2×10^6 cell/ml (OD_{600} = ~0.10). Incubate at 30°C until the culture contains ~1×10^7 cells/ml (OD_{600} = ~0.50).

3. Centrifuge 5 min at 1000 to 1500 × *g* in a low-speed centrifuge at room temperature to harvest cells. Resuspend in 30 ml sterile water and transfer to 50-ml conical tube.

4. Centrifuge 5 min at 1000 to 1500 × *g*. Decant supernatant and resuspend cells in 1.5 ml TE buffer/0.1 M lithium acetate.

5. Add 1 µg library DNA in pJG4-5 and 50 µg high-quality sheared salmon sperm carrier DNA to each of 30 sterile 1.5-ml microcentrifuge tubes. Add 50 µl of the resuspended yeast solution from step 4 to each tube.

   The total volume of library and salmon sperm DNA added should be <20 µl and preferably <10 µl.

   A typical library transformation will result in 2 to 3 × 10^6 primary transformants. Assuming a transformation efficiency of 10^5/µg library DNA, this transformation requires a total of 20 to 30 µg library DNA and 1 to 2 mg carrier DNA. Doing transformations in small aliquots helps reduce the likelihood of contamination, and for reasons that are not clear, provides significantly better transformation efficiency than scaled-up versions.

   Do not use excess transforming library DNA per aliquot of competent yeast cells because each competent cell may take up multiple library plasmids, complicating subsequent analysis.

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6. Add 300 µl of sterile 40% PEG 4000/0.1 M lithium acetate/TE buffer, pH 7.5, and invert to mix thoroughly. Incubate 30 min at 30°C.

7. Add DMSO to 10% (~40 µl per tube) and invert to mix. Heat shock 10 min in 42°C heating block.

8a. *For 28 tubes:* Plate the complete contents of one tube per 24 × 24–cm Glu/CM –Ura, –His, –Trp dropout plate and incubate at 30°C.

8b. *For two remaining tubes:* Plate 360 µl of each tube on 24 × 24–cm Glu/CM –Ura, –His, –Trp dropout plate. Use the remaining 40 µl from each tube to make a series of 1/10 dilutions in sterile water. Plate dilutions on 100-mm Glu/CM –Ura, –His, –Trp dropout plates. Incubate all plates 2 to 3 days at 30°C until colonies appear.

The dilution series gives an idea of the transformation efficiency and allows an accurate estimation of the number of transformants obtained.

Collect primary transformant cells

Conventional replica plating (Treco and Winston, 1992) does not work well in the selection process because so many cells are transferred to new plates that very high background levels inevitably occur. Instead, the procedure described below creates a slurry in which cells derived from $>10^6$ primary transformants are homogeneously dispersed. A precalculated number of these cells is plated for each primary transformant.

9. Cool all of the 24 × 24–cm plates containing transformants for several hours at 4°C to harden agar.

10. Wearing gloves and using a sterile glass microscope slide, gently scrape yeast cells off the plate. Pool cells from the 30 plates into one or two sterile 50-ml conical tubes.

This is the step where contamination is most likely to occur. Be careful.

11. Wash cells by adding a volume of sterile TE buffer or water at least equal to the volume of the transferred cells. Centrifuge ~5 min at 1000 to 1500 × *g*, room temperature, and discard supernatant. Repeat wash.

After the second wash, pellet volume should be ~25 ml cells derived from 1.5×10^6 transformants.

12. Resuspend pellet in 1 vol glycerol solution, mix well, and store up to 1 year in 1-ml aliquots at –70°C.

Determine replating efficiency

13. Remove an aliquot of frozen transformed yeast and dilute 1/10 with Gal/Raff/CM –Ura, –His, –Trp dropout medium. Incubate with shaking 4 hr at 30°C to induce the *GAL* promoter on the library.

Raffinose (Raff) aids in growth without diminishing transcription from the GAL1 promoter.

14. Make serial dilutions of the yeast cells using the Gal/Raff/CM –Ura, –His, –Trp dropout medium. Plate on 100-mm Gal/Raff/CM –Ura, –His, –Trp dropout plates and incubate 2 to 3 days at 30°C until colonies are visible.

15. Count colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

In calculating yeast concentrations, it is useful to remember that 1 OD_{600} unit = $\sim 2.0 \times 10^7$ yeast cells. In general, if the harvest is done carefully, viability will be greater than 90%. Some intrepid investigators perform this step simultaneously with plating out on Leu selective medium (steps 16 and 17).

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Screen for interacting proteins

16. Thaw the appropriate quantity of transformed yeast based on the plating efficiency, dilute, and incubate as in step 13. Dilute cultures in Gal/Raff/CM –Ura, –His, –Trp, –Leu medium as necessary to obtain a concentration of 10^7 cells/ml (OD_{600} = ~0.5), and plate 100 µl on each of as many 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu dropout plates as are necessary for full representation of transformants. Incubate 2 to 3 days at 30°C until colonies appear.

    Because not all cells that contain interacting proteins plate at 100% efficiency on –Leu medium (Estojak et al., 1995), it is desirable that for actual selection, each primary colony obtained from the transformation be represented on the selection plate by three to ten individual yeast cells. This will in some cases lead to multiple isolations of the same cDNA; however, because the slurry is not perfectly homogenous, it will increase the likelihood that all primary transformants are represented by at least one cell on the selective plate.

    It is easiest to visually scan for Leu$^+$ colonies using cells plated at ~10^6 cfu per 100-mm plate. Plating at higher density can contribute to cross-feeding between yeast, resulting in spurious background growth. Thus, for a transformation in which 3×10^6 colonies are obtained, plate ~2×10^7 cells on a total of 20 selective plates.

17. Carefully pick appropriate colonies to a new Gal/Raff/CM –Ura, –His, –Trp, –Leu master dropout plate. Incubate 2 to 7 days at 30°C until colonies appear.

    A good strategy is to pick a master plate with colonies obtained on day 2, a second master plate (or set of plates) with colonies obtained on day 3, and a third with colonies obtained on day 4. Colonies from day 2 and 3 master plates should generally be characterized further. If many apparent positives are obtained, it may be worth making master plates of the much larger number of colonies likely to be obtained at day 4 (and after). See Critical Parameters and annotation to step 19 for additional information about appropriate colony selection for the master plate.

    If no colonies appear within a week, those arising at later time points are likely to be artifactual. Contamination that has occurred at an earlier step (e.g., during plate scraping) is generally reflected by the growth of a very large number of colonies (>500/plate) within 24 to 48 hr after plating on selective medium.

    Some investigators omit use of a Gal/Raff/CM –Ura, –His, –Trp, –Leu master plate, restreaking directly to a Glu/CM –Ura, –His, –Trp master plate as in step 19.

Test for Gal dependence

The following steps test for Gal dependence of the Leu$^+$ insert and lacZ phenotypes to confirm that they are attributable to expression of the library-encoded proteins. The *GAL1* promoter is turned off and –Leu selection eliminated before reinducing.

18. Restreak from the Gal/Raff/CM –Ura, –His, –Trp, –Leu master dropout plate to a 100-mm Glu/CM –Ura, –His, –Trp master dropout plate. Incubate overnight at 30°C until colonies form.

19. Restreak or replica plate from this plate to the following plates:

    Glu/Xgal/CM –Ura, –His, –Trp
    Gal/Raff/Xgal/CM –Ura, –His, –Trp
    Glu/CM –Ura, –His, –Trp, –Leu
    Gal/Raff/CM –Ura, –His, –Trp, –Leu.

    At this juncture, colonies and the library plasmids they contain are tentatively considered positive if they are blue on Gal/Raff/Xgal plates but not blue or only faintly blue on Glu/Xgal plates, and if they grow on Gal/Raff/CM –Leu plates but not on Glu/CM –Leu plates.

    The number of positives obtained will vary drastically from bait to bait. How they are processed subsequently will depend on the number initially obtained and on the preference

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of the individual investigator. If none are obtained using EGY48 as reporter strain, it may be worth attempting to screen a library from an additional tissue source. If a relatively small number (≤30) are obtained, proceed to step 20. However, sometimes searches will yield large numbers of colonies (>30 to 300, or more). In this case, there are several options. The first option is to warehouse the majority of the positives and work up the first 30 that arise; those growing fastest are frequently the strongest interactors. These can be checked for specificity, and restriction digests can be used to establish whether they are all independent cDNAs or represent multiple isolates of the same, or a small number, of cDNAs. If the former is true, it may be advisable to repeat the screen in a less sensitive strain background, as obtaining many different interactors can be a sign of low-affinity nonspecific background. Alternatively, if initial indications are that a few cDNAs are dominating the positives obtained, it may be useful to perform a filter hybridization with yeast (see Support Protocol 3) using these cDNAs as a probe to establish the frequency of their identification and exclude future reisolation of these plasmids. The second major option is to work up large numbers of positives to get a complete profile of isolated interactors (see Support Protocol 4). A third option is to temporarily warehouse the entire results of this first screen, and repeat the screen with a less sensitive strain such as EGY191, on the theory that it is most important to get stronger interactors first and a complete profile of interactors later. Finally, some investigators prefer to work up the entire set of positives initially obtained, even if such positives number in the hundreds. Particularly in this latter case, it is most effective to use Alternate Protocol 1 as a means to identify unique versus common positives.

Isolate plasmid from positive colonies by transfer into E. coli

20a. Transfer yeast plasmids directly into *E. coli* by following the protocol for direct electroporation (*UNIT 5.10*). Proceed to step 22.

20b. Isolate plasmid DNA from yeast by the rapid miniprep protocol (*APPENDIX 4C*) with the following alteration: after obtaining aqueous phase, precipitate by adding sodium acetate to 0.3 M final and 2 vol ethanol, incubate 20 min on ice, microcentrifuge 15 min at maximum speed, wash pellet with 70% ethanol, dry, and resuspend in 5 µl TE buffer.

Cultures can be grown prior to the miniprep using Glu/CM –Trp to select only for the library plasmid; this may increase the proportion of bacterial colonies that contain the desired plasmid.

21. Use 1 µl DNA to electroporate (*UNIT 5.10*) into competent KC8 bacteria, and plate on LB/ampicillin plates. Incubate overnight at 37°C.

Electroporation must be used to obtain transformants with KC8 because the strain is generally refractory to transformation.

22. Restreak or replica plate colonies arising on LB/ampicillin plates to bacterial defined minimal A medium plates containing vitamin B1 and supplemented with Ura, His, and Leu but lacking Trp. Incubate overnight at 37°C.

Colonies that grow under these conditions contain the library plasmid.

The yeast TRP1 gene can successfully complement the bacterial trpC-9830 mutation, allowing the library plasmid to be easily distinguished from the other two plasmids contained in the yeast. It is helpful to first plate transformations on LB/ampicillin plates, which provides a less stringent selection, followed by restreaking to bacterial minimal medium to maximize the number of colonies obtained (E.G., unpub. observ.).

23. Purify library-containing plasmids using a bacterial miniprep procedure (*APPENDIX 4C*).

Some investigators are tempted to immediately sequence DNAs obtained at this stage. At this point, it is still possible that none of the isolated clones will express bona fide

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interactors, and it is suggested that the following specificity tests be completed before committing the effort to sequencing (also see annotation to step 28).

Because multiple 2μm plasmids with the same marker can be simultaneously tolerated in yeast, it sometimes happens that a single yeast will contain two or more different library plasmids, only one of which encodes an interacting protein. The frequency of this occurrence varies in the hands of different investigators and may in some cases account for disappearing positives if the wrong cDNA is picked. When choosing colonies to miniprep, it is generally useful to work up at least two individual bacterial transformants for each yeast positive. These minipreps can then be restriction digested (APPENDIX 4I) with EcoRI + XhoI to release cDNA inserts, and the size of inserts determined on an agarose minigel (APPENDIX 4F) to confirm that both plasmids contain the same insert. An additional benefit of analyzing insert size is that it may provide some indication as to whether repeated isolation of the same cDNA is occurring, generally a good indication concerning the biological relevance of the interactor. See Background Information for further discussion.

Assess positive colonies with specificity tests

Much spurious background will have been removed by the previous series of controls. Other classes of false positives can be eliminated by retransforming purified plasmids into "virgin" *LexA*-operator-*LEU2*/*LexA*-operator-*lacZ*/pBait–containing strains that have not been subjected to Leu selection and verifying that interaction-dependent phenotypes are still observed. Such false positives could include mutations in the initial EGY48 yeast that favor growth on Gal medium, library-encoded cDNAs that interact with the LexA DNA-binding domain, or proteins that are sticky and interact with multiple biologically unrelated fusion domains.

24. In separate transformations, use purified plasmids from step 23 to transform yeast that already contain the following plasmids and are growing on Glu/CM –Ura, –His plates:

    EGY48 containing pSH18-34 and pBait
    EGY48 containing pSH18-34 and pRFHM-1
    EGY48 containing pSH18-34 and a nonspecific bait (optional).

25. Plate each transformation mix on Glu/CM –Ura, –His, –Trp dropout plates and incubate 2 to 3 days at 30°C until colonies appear.

26. Create a Glu/CM –Ura, –His, –Trp master dropout plate for each library plasmid being tested. Streak adjacently five or six independent colonies derived from each of the transformation plates. Incubate overnight at 30°C.

27. Restreak or replica plate from this master dropout plate to the same series of test plates used for the actual screen:

    Glu/Xgal/CM –Ura, –His, –Trp
    Gal/Raff/Xgal/CM –Ura, –His, –Trp
    Glu/CM –Ura, –His, –Trp, –Leu
    Gal/CM –Ura, –His, –Trp, –Leu.

True positive cDNAs should make cells blue on Gal/Raff/Xgal but not on Glu/Xgal plates, and should make them grow on Gal/Raff/CM –Leu but not Glu/CM –Leu dropout plates only if the cells contain LexA-bait. cDNAs that meet such criteria are ready to be sequenced (see legend to Fig. 19.2.3 for primer sequence) or otherwise characterized. Those cDNAs that also encode proteins that interact with either RFHM-1 or another nonspecific bait should be discarded.

It may be helpful to cross-check the isolated cDNAs with a database of cDNAs thought to be false positives. This database is available on the World Wide Web as a work in progress at http://www.fccc.edu:80/research/labs/golemis/InteractionTrapInWork.html. cDNAs reported to this database are generally those isolated only once in a screen in which obviously

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true interactive partners were isolated multiple times, cDNAs that may interact with more than one bait, or cDNAs for which the interaction does not appear to make biological sense in the context of the starting bait. Although some proteins in this database may ultimately turn out in fact to associate with the bait that isolated them, they are by default unlikely to possess a unique and interesting function in the context of that bait if they are well represented in the database.

28. If appropriate, conduct additional specificity tests (see Support Protocol 5). Analyze and sequence positive isolates.

The primer sequence for use with pJG4-5 is provided in the legend to Figure 19.2.4.

DNA prepared from KC8 is generally unsuitable for dideoxy or automated sequencing even after use of Qiagen columns and/or cesium chloride gradients. Library plasmids to be sequenced should be retransformed from the KC8 miniprep stock (step 23) to a more amenable strain, such as DH5α, before sequencing is attempted.

RAPID SCREEN FOR INTERACTION TRAP POSITIVES

ALTERNATE PROTOCOL 1

Under some circumstances, it may be desirable to attempt the analysis of a large number of positives resulting from a two-hybrid screen. One such hypothetical example would be a bait with a leucine zipper or coiled coil known to dimerize with partner "A" that is highly expressed. In order to identify the rare novel partner "B", it is necessary to work through the high background of "A" reisolates. This protocol uses the polymerase chain reaction (PCR) in a strategy to sort positives into redundant (multiple isolates) and unique classes prior to plasmid rescue from yeast, thus greatly reducing the number of plasmid isolations that must be performed. An additional benefit is that this protocol preidentifies positive clones containing one or multiple library plasmids; for those containing only one library plasmid, only a single colony needs to be prepared through KC8/DH5α.

Additional Materials (also see Basic Protocol 2)

Yeast plated on Glu/CM –Ura, –His, –Trp master plate (see Basic Protocol 2, step 19)
Lysis solution (see recipe)
10 μM forward primer (FP1): 5′-CGT AGT GGA GAT GCC TCC-3′
10 μM reverse primer (FP2): 5′-CTG GCA AGG TAG ACA AGC CG-3′

Toothpicks or bacterial inoculating loops (*APPENDIX 4A*), sterile
96-well microtiter plate
Sealing tape, e.g., wide transparent tape
150- to 212-μm glass beads, acid-washed
Vortexer with flat plate

Additional reagents and equipment for performing an interactor hunt (see Basic Protocol 2), PCR amplification of DNA (*APPENDIX 4J*), agarose gel electrophoresis (*APPENDIX 4F*), restriction endonuclease digestion (*APPENDIX 4I*), electroporation (*UNIT 5.10*), and miniprep isolation of bacterial DNA (*APPENDIX 4C*)

1. Perform an interactor hunt (see Basic Protocol 2, steps 1 to 19).
2. Use a sterile toothpick or bacterial inoculating loop to transfer yeast from the Glu/CM, –Ura, –His, –Trp master plate into 25 μl lysis solution in a 96-well microtiter plate. Seal the wells of the microtiter plate with sealing tape and incubate 1.5 to 3.5 hr at 37°C with shaking.

The volume of yeast transferred should not exceed ~2 to 3 μl of packed pellet; larger quantities of yeast will reduce quality of the DNA. DNA can be efficiently recovered from master plates that have been stored up to 1 week at 4°C. If yeast have been previously

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gridded on master plates, transfer to microtiter plates can be facilitated by using a multicolony replicator.

3. Remove tape from the plate, add ~25 µl acid-washed glass beads to each well, and reseal with the same tape. Firmly attach the microtiter plate to a flat-top vortexer, and vortex 5 min at medium-high power.

   The microtiter plate can be attached to the vortexer using 0.25-in (0.64-cm) rubber bands.

4. Remove the tape and add ~100 µl sterile water to each well. Swirl gently to mix, then remove sample for step 5. Press the tape back firmly to seal the microtiter plate and place in the freezer at –20°C for storage.

5. Amplify 0.8 to 2.0 µl of sample by standard PCR (*APPENDIX 4J*) in a ~30-µl volume using 3 µl each of the forward primer FP1 and the reverse primer FP2. Perform PCR using the following cycles:

Initial step:	2 min	94°C
31 cycles:	45 sec	94°C
	45 sec	56°C
	45 sec	72°C.

   These conditions have been used successfully to amplify fragments up to 1.8 kb in length; some modifications, such as extension of elongation time, are also effective.

6. Load 20 µl of the PCR reaction product on a 0.7% low melting temperature agarose gel (*APPENDIX 4F*) to resolve PCR products. Based on insert sizes, group the obtained interactors in families, i.e., potential multiple independent isolates of identical cDNAs. Reserve gel until results of step 7 are obtained.

   No special precautions are needed for storing the gel. Since HaeIII digests typically yield rather small DNA fragments, running the second gel does not take a lot of time. Usually, the delay does not exceed 45 to 60 min, during which time the first gel may be stored in a gel box at room temperature or wrapped in plastic wrap at 4°C.

7. While the gel is running, use the remaining 10 µl of PCR reaction product for a restriction endonuclease digestion with *Hae*III in a digestion volume of ~20 µl (*APPENDIX 4I*). Based on analysis of the sizes of undigested PCR products in the gel (step 6), rearrange the tubes with *Hae*III digest samples so that those thought to represent a family are side by side. Resolve the digests on a 2% to 2.5% agarose gel (*APPENDIX 4F*).

   Most restriction fragments will be in the 200-bp to 1.0-kb size range so using a long gel run is advisable. This analysis should produce a distinct fingerprint of insert sizes and allow definition of library cDNAs as unique isolates or related groups.

   A single positive yeast will sometimes contain multiple library plasmids. An advantage of this protocol is the ready detection of multiple library plasmids in PCR reactions; thus, following subsequent bacterial transformations, only a single TRP1 colony would need to be analyzed unless multiple plasmids were already known to be present.

8. Isolate DNA fragments from the low melting temperature agarose gel (step 6).

   If inspection of the banding pattern on the two gels suggests that a great many reisolates of a small number of cDNAs are present, it may be worthwhile to immediately sequence PCR products representative of these clusters, but it is generally still advisable to continue through specificity tests before doing so. If the PCR products are sequenced, the FP1 forward primer works well in automated sequencing of PCR fragments, but the FP2 primer is only effective in sequencing from purified plasmid.

   In general, priming from the AT-rich ADH terminator downstream of the polylinker/cDNA in library plasmid is less efficient than from upstream of the cDNA, and it is hard to design effective primers in this region.

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9. Remove the microtiter plate of lysates from the freezer, thaw it, and remove 2 to 4 μl of lysed yeast for each desired positive. Electroporate DNA into either DH5α or KC8 *E. coli* as appropriate, depending on the choice of bait and reporter plasmids (see Table 19.2.1 and see Background Information for further information). Refreeze the plate as a DNA reserve in case bacteria fail to transform on the first pass.

   KC8 E. coli should be used for electroporation when the original reagents pEG202/pJG4-5/pJK101 are used for the interaction trap.

   An additional strength of this protocol is that it identifies redundant clones before transfer of plasmids to bacteria, thus reducing the amount of work required in cases where plasmid identity can be unambiguously assigned. However, although restriction endonuclease digestion and PCR analysis are generally highly predictive, they are not 100% certain methods for estimating cDNA identity. Thus, if there is any doubt about whether two cDNAs are the same, investigators are urged to err on the side of caution.

10. Prepare a miniprep of plasmid DNA from the transformed bacteria (*APPENDIX 4C*) and perform yeast transformation and specificity assessment (see Basic Protocol 2, steps 24 to 28).

PERFORMING A HUNT BY INTERACTION MATING

ALTERNATE PROTOCOL 2

An alternative way of conducting an interactor hunt is to mate a strain that expresses the bait protein with a strain that has been pretransformed with the library DNA, and screen the resulting diploid cells for interactors (Bendixen et al., 1994; Finley and Brent, 1994). This "interaction mating" approach can be used for any interactor hunt, and is particularly useful in three special cases. The first case is when more than one bait will be used to screen a single library. Interaction mating allows several interactor hunts with different baits to be conducted using a single high-efficiency yeast transformation with library DNA. This can be a considerable savings, since the library transformation is one of the most challenging tasks in an interactor hunt. The second case is when a constitutively expressed bait interferes with yeast viability. For such baits, performing a hunt by interaction mating avoids the difficulty associated with achieving a high-efficiency library transformation of a strain expressing a toxic bait. Moreover, the actual selection for interactors will be conducted in diploid yeast, which are more vigorous than haploid yeast and can better tolerate expression of toxic proteins. The third case is when a bait cannot be used in a traditional interactor hunt using haploid yeast strains (see Basic Protocol 2) because it activates transcription of even the least sensitive reporters. In diploids the reporters are less sensitive to transcription activation than they are in haploids. Thus, the interaction mating hunt provides an additional method to reduce background from transactivating baits.

In the protocol described below, the library DNA is used to transform a strain with a *LEU2* reporter (e.g., EGY48). This pretransformed library strain is then frozen in many aliquots, which can be thawed and used for individual interactor hunts. The bait is expressed in a strain of mating type opposite to that of the pretransformed library strain, and also bearing the *lacZ* reporter. A hunt is conducted by mixing the pretransformed library strain with the bait strain and allowing diploids to form on YPD medium overnight. The diploids are then induced for expression of the library-encoded proteins and screened for interactors as in Basic Protocol 2.

NOTE: Strain combinations other than those described below can also be used in an interaction-mating hunt. The key to choosing the strains is to ensure that the bait and prey strains are of opposite mating types and that both have auxotrophies to allow selection for the appropriate plasmids and reporter genes. Also, once the bait plasmid and *lacZ* reporter plasmid have been introduced into the bait strain, and the library plasmids have

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been introduced into the library strain, the resulting bait strain and library strain must each have auxotrophies that can be complemented by the other, so that diploids can be selected.

Additional Materials (also see Basic Protocols 1 and 2)

Yeast strains: either RFY206 (Finley and Brent, 1994), YPH499 (Sikorski and Hieter, 1989; ATCC #6625), or an equivalent *MATa* strain with auxotrophic markers *ura3*, *trp1*, *his3*, and *leu2*
YPD liquid medium (*APPENDIX 4L*)
Glu/CM –Trp plates: CM dropout plates –Trp (*APPENDIX 4L*) supplemented with 2% glucose
pJG4-5 library vector (Fig. 19.2.6), empty
100-mm YPD plates (*APPENDIX 4L*)

Additional reagents and equipment for lithium acetate transformation of yeast (*APPENDIX 4L*)

Construct the bait strain

The bait strain will be a *MATa* yeast strain (mating type opposite of EGY48) containing a *lacZ* reporter plasmid like pSH18-34 and the bait-expressing plasmid, pBait.

1. Perform construction of the bait plasmid (pBait; see Basic Protocol 1, step 1).

2. Cotransform the *MATa* yeast strain (e.g., either RFY206 or YPH499) with pBait and pSH18-34 using the lithium acetate method (*APPENDIX 4L*). Select transformants on Glu/CM –Ura,–His plates by incubating plates at 30°C for 3 to 4 days until colonies form. Combine 3 colonies for all future tests and for the mating hunt.

   The bait strain (RFY206/pSH18-34/pBait or YPH499/pSH18-34/pBait) can be tested by immunoblotting to ensure that the bait protein is expressed (see Support Protocol 1). Synthesis and nuclear localization of the bait protein can also be tested by the repression assay (see Basic Protocol 1, steps 8 to 12).

3. *Optional:* Assay *lacZ* gene activation in the bait strain (see Basic Protocol 1, steps 4 to 7).

   If the bait activates the lacZ reporter, a less sensitive lacZ reporter plasmid (Table 19.2.1), or an integrated version of the lacZ reporter should be tried. A bait that strongly activates the lacZ reporters usually cannot be used in a hunt based on selection of interactors with the LEU2 reporter, because the LEU2 reporters are more sensitive than the lacZ reporters. However, both reporters are less sensitive to activation by a bait in diploid cells, as compared to haploid cells. Thus, a more important test of the transactivation potential of a bait is to test the leucine requirement of diploid cells expressing it, as described in steps 6 to 20, below.

Prepare the pretransformed library strain (EGY48 + library plasmids)

4. Perform a large-scale transformation of EGY48 with library DNA using the lithium acetate method (see Basic Protocol 2, steps 1 to 8, except start with EGY48 bearing no other plasmids). To prepare for transformation, grow EGY48 in YPD liquid medium. Select library transformants on Glu/CM –Trp plates by incubating 3 days at 30°C.

5. Collect primary transformants by scraping plates, washing yeast, and resuspending in 1 pellet vol glycerol solution (see Basic Protocol 2, steps 9 to 12). Freeze 0.2 to 1.0 ml aliquots at –70° to –80°C.

   The cells will be stable for at least 1 year. Refreezing a thawed aliquot will result in loss of viability. Thus, many frozen aliquots should be made, so that each thawed aliquot can be discarded after use.

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Prepare the pretransformed control strain (EGY48 + pJG4-5)

6. Transform EGY48 grown in YPD liquid medium with the empty library vector, pJG4-5, using the lithium acetate method (*APPENDIX 4L*). Select transformants on Glu/CM –Trp plates by incubating 3 days at 30°C.

7. Pick and combine three transformant colonies and use them to inoculate 30 ml of Glu/CM –Trp medium. Incubate 15 to 24 hr at 30°C (to OD_{600} >3).

8. Centrifuge 5 min at 1000 to 1500 × *g*, room temperature, and remove supernatant. Resuspend in 10 ml sterile water to wash cells.

9. Centrifuge 5 min at 1000 to 1500 × *g*, room temperature, and remove supernatant. Resuspend in 1 pellet vol glycerol solution and freeze 100-µl aliquots at –70° to –80°C.

Determine plating efficiency of pretransformed library and pretransformed control strains

10. After freezing (at least 1 hr) thaw an aliquot of each pretransformed strain (from step 5 and step 9) at room temperature. Make several serial dilutions in sterile water, including aliquots diluted 10^5-fold, 10^6-fold, and 10^7-fold. Plate 100 µl of each dilution on 100-mm Glu/CM –Trp plates and incubate 2 to 3 days at 30°C.

11. Count the colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

    The plating efficiency for a typical library transformation and for the control strain will be ~1 × 10^8 cfu per 100 µl.

Mate the bait strain with the pretransformed library strain and the pretransformed control strain

In steps 12 through 20, an interactor hunt is conducted concurrently with testing *LEU2* reporter activation by the bait itself. For most baits, this approach will be the quickest way to isolate interactors. However, for some baits, such as those that have a high transactivation potential, or those that affect yeast mating or growth, steps 12 through 20 will serve as a pilot experiment to determine the optimal parameters for a subsequent hunt.

12. Grow a 30-ml culture of the bait strain in Glu/CM –Ura,–His liquid dropout medium to mid to late log phase (OD_{600} = 1.0 to 2.0, or 2 to 4 × 10^7cells/ml).

    A convenient way to grow the bait strain is to inoculate a 5-ml culture with approximately three colonies from a plate and grow it overnight at 30°C with shaking. In the morning, measure the OD_{600}, dilute into a 30-ml culture to a final OD_{600} = 0.2, and grow at 30°C with shaking. The culture should reach mid to late log phase before the end of the day.

13. Centrifuge the culture 5 min at 1000 to 1500 × *g*, room temperature, to harvest cells. Resuspend the cell pellet in sterile water to make a final volume of 1 ml.

    This should correspond to ~1 × 10^9 cells/ml.

14. Set up two matings. In one sterile microcentrifuge tube mix 200 µl of the bait strain with 200 µl of a thawed aliquot of the pretransformed control strain from step 9. In a second microcentrifuge tube mix 200 µl of the bait strain with ~1 × 10^8 cfu (~0.1 to 1 ml) of the pretransformed library strain from step 5.

    The library mating should be set up so that it contains a ~2-fold excess of bait strain cfu over pretransformed library strain cfu. Because the bait strain was harvested in log phase, most of the cells will be viable (i.e., cells/ml = ~cfu/ml), and the number of cfu can be sufficiently estimated from optical density (1 OD_{600} = ~2 × 10^7 cells/ml). Under these conditions, ~10% of the cfu in the pretransformed library strain will mate with the bait

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strain. Thus, a complete screen of 10^7 library transformants will require a single mating with at least 10^8 cfu of the pretransformed library strain and at least 2×10^8 cfu of the bait strain.

To screen more library transformants, set up additional matings. The number of pretransformed library transformants to screen depends on the size of the library and the number of primary transformants obtained in step 5. If the size of the library is larger than the number of transformants obtained in step 5, the goal will be to screen all of the yeast transformants. In this case, complete screening of the library will require additional transformations of EGY48 and additional interactor hunts. If the size of the library is smaller than the number of transformants obtained in step 5, the goal will be to screen at least a number of transformants equivalent to the size of the library.

15. Centrifuge each cell mixture for 5 min at 1000 to 1500 × *g*, pour off medium, and resuspend cells in 200 µl YPD medium. Plate each suspension on a 100-mm YPD plate. Incubate 12 to 15 hr at 30°C.

16. Add ~1 ml of Gal/Raff/CM –Ura, –His, –Trp to the lawns of mated yeast on each plate. Mix the cells into the medium using a sterile applicator stick.

17. Transfer each slurry of mated cells to a 500-ml flask containing 100 ml of Gal/Raff/CM –Ura, –His, –Trp dropout medium. Incubate with shaking 6 hr at room temperature to induce the *GAL1* promoter, which drives expression of the cDNA library.

18. Centrifuge the cell suspensions 5 min at 1000 to 1500 × *g*, room temperature, to harvest the cells. Wash by resuspending in 30 ml of sterile water and centrifuging again. Resuspend each pellet in 5 ml sterile water. Measure OD_{600} and, if necessary, dilute to a final concentration of $\sim 1 \times 10^8$ cells/ml.

This is a mixture consisting of haploid cells that have not mated and diploid cells. Under a microscope, the two cell types can be distinguished by size (diploids are ~1.7× bigger than haploids) and shape (diploids are slightly oblong and haploids are spherical). Because diploids grow faster than haploids, this mixture will contain ~10% to 50% diploid cells. The actual number of diploids will be determined by plating dilutions on –Ura, –His, –Trp medium, which will not support the growth of the parental haploids.

19. For each mating make a series of $1/10$ dilutions in sterile water, at least 200 µl each, to cover a 10^6-fold concentration range. Plate 100 µl from each tube (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilution) on 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Plate 100 µl from the 10^{-4}, 10^{-5}, and 10^{-6} tubes on 100-mm Gal/Raff/CM –Ura, –His, –Trp plates. Incubate plates at 30°C. Count the colonies on each plate after 2 to 5 days.

20. For the mating with the pretransformed library, prepare an additional 3 ml of a 10^{-1} dilution. Plate 100 µl of the 10^{-1} dilution on each of 20 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Also plate 100 µl of the undiluted cells on each of 20 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Incubate at 30°C. Pick Leu^+ colonies after 2 to 5 days and characterize them beginning with step 17 of Basic Protocol 2.

The number of Leu^+ colonies to pick to ensure that all of the pretransformed library has been screened depends on the transactivation potential of the bait protein itself. The transactivation potential is expressed as the number of Leu^+ colonies that grow per cfu (Leu^+/cfu) of the bait strain mated with the control strain, as determined in step 19 of this protocol. It can be calculated as the ratio of the number of colonies that grow on Gal/Raff/CM –Ura,–His, –Trp, –Leu to the number of colonies that grow on Gal/Raff/CM –Ura, –His, –Trp for a given dilution of the mating between the bait strain and the control strain. A bait with essentially no transactivation potential will produce less than 10^{-6} Leu^+/cfu. For a bait to be useful in an interactor hunt it should not transactivate more than 10^{-4} Leu^+/cfu.

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To screen all of the pretransformed library, it will be necessary to pick a sufficient number of Leu$^+$ colonies in addition to background colonies produced by the transactivation potential of the bait itself. Thus, the minimum number of Leu$^+$ colonies that should be picked in step 20 of this protocol is given by:

(transactivation potential, Leu$^+$/cfu) × (# library transformants screened).

For example, if 10^7 library transformants were obtained in step 2 (and at least 10^8 cfu of these transformants were mated with the bait strain in step 14, since only ~10% will form diploids), and the transactivation potential of the bait is 10^{-4} Leu$^+$/cfu, then at least 1000 Leu$^+$ colonies must be picked and characterized. In other words, if the rarest interactor is present in the pretransformed library at a frequency of 10^{-7}, to find it one needs to screen through at least 10^7 diploids from a mating of the library strain. However, at least 1000 of these 10^7 diploids would be expected to be Leu$^+$ due to the bait background if the transactivation potential of the bait is 10^{-4}. The true positives will be distinguished from the bait background in the next step by the galactose dependence of their Leu$^+$ and lacZ$^+$ phenotypes.

PREPARATION OF PROTEIN EXTRACTS FOR IMMUNOBLOT ANALYSIS

SUPPORT PROTOCOL 1

To confirm that the bait fusion protein constructed in Basic Protocol 1 is synthesized properly, a crude lysate is prepared for SDS-PAGE and immunoblot analysis (*UNITS 10.1 & 10.10*). The presence of the target protein is detected by antibody to LexA or the fusion domain.

Materials

Master plates with pBait-containing positive and control yeast on Glu/CM –Ura, –His dropout medium (see Basic Protocol 1, step 4)

Glu/CM –Ura, –His dropout liquid medium: CM dropout plates –Ura, –His (*APPENDIX 4L*) supplemented with 2% glucose

2× Laemmli sample buffer (see recipe)

Antibody to fusion domain or LexA: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

30°C incubator

100°C water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 10.1*) and immunoblotting and immunodetection (*UNIT 10.10*)

1. From the master plates, start a 5-ml culture in Glu/CM –Ura, –His liquid medium for each bait being tested and for a positive control for protein expression (i.e., RFHMI or SH17-4). Incubate overnight at 30°C.

   For each construct assayed, it is a good idea to grow colonies from at least two primary transformants, as levels of bait expression are sometimes heterogenous.

2. From each overnight culture, start a fresh 5-ml culture in Glu/CM –Ura, –His at OD_{600} = ~0.15. Incubate again at 30°C.

3. When the culture has reached OD_{600} = 0.45 to 0.7 (~4 to 6 hr), remove 1.5 ml to a microcentrifuge tube.

   For some LexA fusion proteins, levels of the protein drop off rapidly in cultures approaching stationary phase. This is due to a combination of the diminishing activity of the ADH1 promoter in late growth phases and the relative instability of particular fusion domains. Thus, it is not a good idea to let cultures become saturated in the hopes of obtaining a higher yield of protein.

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4. Microcentrifuge cells 3 min at 13,000 × *g*, room temperature. When the pellet is visible, remove the supernatant.

   Inspection of the tube should reveal a pellet ~1 to 3 µl in volume. If the pellet is not visible, microcentrifuge another 3 min.

5. Working rapidly, add 50 µl of 2× Laemmli sample buffer to the visible pellet in the tube, vortex, and place the tube on dry ice.

   Samples may be frozen at –70°C.

6. Transfer frozen sample directly to a boiling water bath or a PCR machine set to cycle at 100°C. Boil 5 min.

7. Microcentrifuge 5 sec at maximum speed to pellet large cellular debris.

8. Perform SDS-PAGE (*UNIT 10.1*) using 20 to 50 µl sample per lane.

9. To detect the protein, immunoblot and analyze (*UNIT 10.10*) using antibody to the fusion domain or LexA.

SUPPORT PROTOCOL 2

PREPARATION OF SHEARED SALMON SPERM CARRIER DNA

This protocol generates high-quality sheared salmon sperm DNA (sssDNA) for use as carrier in transformation (Basic Protocol 2). This DNA is also suitable for other applications where high-quality carrier DNA is needed (e.g., hybridization). This protocol is based on Schiestl and Gietz (1989). For more details of phenol extraction or other DNA purification methods, consult *APPENDIX 4E*.

Materials

High-quality salmon sperm DNA (e.g., sodium salt from salmon testes, Sigma or Boehringer Mannheim), desiccated
TE buffer, pH 7.5 (*APPENDIX 2E*), sterile
TE-saturated buffered phenol (*APPENDIX 2E*)
1:1 (v/v) buffered phenol/chloroform
Chloroform
3 M sodium acetate, pH 5.2 (*APPENDIX 2E*)
100% and 70% ethanol, ice cold

Magnetic stirring apparatus and stir-bar, 4°C
Sonicator with probe
50-ml conical centrifuge tube
High-speed centrifuge and appropriate tube
100°C and ice-water baths

1. Dissolve desiccated high-quality salmon sperm DNA in TE buffer, pH 7.5, at a concentration of 5 to 10 mg/ml by pipetting up and down in a 10-ml glass pipet. Place in a beaker with a stir-bar and stir overnight at 4°C to obtain a homogenous viscous solution.

   It is important to use high-quality salmon sperm DNA. Sigma Type III sodium salt from salmon testes has worked well, as has a comparable grade from Boehringer Mannheim. Generally it is convenient to prepare 20- to 40-ml batches at a time.

2. Shear the DNA by sonicating briefly using a large probe inserted into the beaker.

   The goal of this step is to generate sheared salmon sperm DNA (sssDNA) with an average size of 7 kb, but ranging from 2 to 15 kb. Oversonication (such that the average size is closer to 2 kb) drastically decreases the efficacy of carrier in enhancing transformation. The original version of this protocol (Schiestl and Gietz, 1989) called for two 30-sec pulses at

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Table 19.2.4 Two-hybrid System Variants[a]

System	DNA-binding domain	Activation domain	Selection	Reference
Two-hybrid	*GAL4*	*GAL4*	Activation of *lacZ*, *HIS3*	Chien et al., 1991
Interaction trap	*LexA*	*B42*	Activation of *LEU2*, *lacZ*	Gyuris et al., 1993
"Improved two-hybrid"	*GAL4*	*GAL4*	Activation of *HIS3*, *lacZ*	Durfee at al., 1993
Modified two-hybrid	*LexA*	VP16	Activation of *HIS3*, *lacZ*	Vojtek at al., 1993
KISS	*GAL4*	VP16	Activation of *CAT*, *hyg*^{r}	Fearon et al., 1992
Contingent replication	*GAL4*	VP16	Activation of T-Ag, replication of plasmids	Vasavada et al., 1991

[a]Abbreviations: *CAT*, chloramphenicol transferase gene; *hyg*^{r}, hygromycin resistance gene; T-Ag, viral large T antigen.

three-quarter power, but optimal conditions vary between sonicators. The first time this protocol is performed, it is worthwhile to sonicate briefly, then test the size of the DNA by running out a small aliquot alongside molecular weight markers on an agarose gel containing ethidium bromide. The DNA can be sonicated further if needed.

3. Once DNA of the appropriate size range has been obtained, extract the sssDNA solution with an equal volume of TE-saturated buffered phenol in a 50-ml conical tube, shaking vigorously to mix.
4. Centrifuge 5 to 10 min at 3000 × *g*, room temperature, or until clear separation of phases is obtained. Transfer the upper phase containing the DNA to a clean tube.
5. Repeat extraction using 1:1 (v/v) buffered phenol/chloroform, then chloroform alone. Transfer the DNA into a tube suitable for high-speed centrifugation.
6. Precipitate the DNA by adding 1/10 vol of 3 M sodium acetate and 2.5 vol of ice-cold 100% ethanol. Mix by inversion. Centrifuge 15 min at ~12,000 × *g*, room temperature.
7. Wash the pellet with 70% ethanol. Briefly dry either by air drying, or by covering one end of the tube with Parafilm with a few holes poked in and placing the tube under vacuum. Resuspend the DNA in sterile TE buffer at 5 to 10 mg/ml.

   Do not overdry the pellet or it will be very difficult to resuspend.
8. Denature the DNA by boiling 20 min in a 100°C water bath. Then immediately transfer the tube to an ice-water bath.
9. Place aliquots of the DNA in microcentrifuge tubes and store frozen at –20°C. Thaw as needed.

   DNA should be boiled again briefly (5 min) immediately before addition to transformations.

   Before using a new batch of sssDNA in a large-scale library transformation, it is a good idea to perform a small-scale transformation using suitable plasmids to determine the transformation efficiency. Optimally, use of sssDNA prepared in the manner described will yield transformation frequencies of >10^5 colonies/μg input plasmid DNA.

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YEAST COLONY HYBRIDIZATION

This protocol is adapted from a modification of the classic protocol of Grunstein and Hogness (1975; Kaiser et al., 1994). It is primarily useful when a large number of putative interactors has been obtained, and initial minipreps and restriction digests have indicated that many of them derive from a small number of cDNAs; these cDNAs can then be used as probes to screen and eliminate identical cDNAs from the pool.

Materials

Glu/CM –Trp plates: CM dropout plates –Trp (*APPENDIX 4L*) supplemental with 2% glucose
Master dropout plate of yeast positive for Gal dependence (see Basic Protocol 2, step 18)
1 M sorbitol/20 mM EDTA/50 mM DTT (prepare fresh)
1 M sorbitol/20 mM EDTA
0.5 M NaOH
0.5 M Tris·Cl (pH 7.5)/6× SSC (*APPENDIX 2E*)
2× SSC (*APPENDIX 2E*)
100,000 U/ml β-glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma)

82-mm circular nylon membrane, sterile
Whatman 3MM paper
80°C vacuum oven *or* UV cross-linker

Additional reagents and equipment for bacterial filter hybridization (Strauss, 1993; Duby et al., 1988)

1. Place a sterile nylon membrane onto a Glu/CM –Trp dropout plate. From the master dropout plate of Gal-dependent positives, gently restreak positives to be screened onto the membrane and mark the membrane to facilitate future identification of hybridizing colonies. Grow overnight (~12 hr) at 30°C.

   Growth for extended periods of time (i.e., 24 hr) may result in difficulty in obtaining good lysis. It is a good idea to streak positive and negative controls for the cDNAs to be hybridized on the membrane.

2. Remove membrane from plate. Air dry briefly. Incubate ~30 min on a sheet of Whatman 3MM paper saturated with 1 M sorbitol/20 mM EDTA/50 mM DTT.

   Optionally, before commencing chemical lysis, membranes can be placed at –70°C for 5 min, then thawed at room temperature for one or more cycles to enhance cell wall breakage.

3. Cut a piece of Whatman 3MM paper to fit inside a 100-mm petri dish. Place the paper disc in the dish and saturate with 100,000 U/ml β-glucuronidase diluted 1:500 in 1 M sorbitol/20 mM EDTA (2 µl glucuronidase per ml of sorbitol/EDTA to give 200 U/ml final). Layer nylon membrane on dish, cover dish, and incubate up to 6 hr at 37°C until >80% of the cells lack a cell wall.

   The extent of cell wall removal can be determined by removing a small quantity of cells from the filter to a drop of 1 M sorbitol/20 mM EDTA on a microscope slide and observing directly with a phase-contrast microscope at ≥60× magnification. Cells lacking cell wall are nonrefractile.

4. Place membrane on Whatman 3MM paper saturated with 0.5 M NaOH for ~8 to 10 min.

5. Place membrane on Whatman 3MM paper saturated with 0.5 M Tris·Cl (pH 7.5)/6× SSC for 5 min. Repeat with a second sheet of Whatman 3MM paper.

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6. Place membrane on Whatman 3MM paper saturated with 2× SSC for 5 min. Then place membrane on dry Whatman paper to air dry for 10 min.
7. Bake membrane 90 min at 80°C in vacuum oven or UV cross-link.
8. Process as for bacterial filter hybridization (Strauss, 1993; Duby et al., 1988), hybridizing the membrane with probes complementary to previously isolated cDNAs.

When selecting probes, either random-primed cDNAs or oligonucleotides complementary to the cDNA sequence may be used. If the cDNA is a member of a protein family, it may be advantageous to use oligonucleotides to avoid inadvertently excluding genes related but not identical to those initially obtained.

MICROPLATE PLASMID RESCUE

SUPPORT PROTOCOL 4

In some cases, it is desirable to isolate plasmids from a large number of positive colonies (Basic Protocol 2, steps 18 and 19). The protocol described below is a batch DNA preparation protocol developed by Steve Kron (University of Chicago, Chicago, Ill.) as a scale-up of a basic method developed by Manuel Claros (Laboratoire de Génétique Moleculaire, Paris, France).

Materials

2× Glu/CM –Trp liquid medium: 2× CM –Trp liquid medium (*APPENDIX 4L*) supplemented with 4% glucose
Master plate of Gal-dependent yeast colonies (see Basic Protocol 2, step 18)
Rescue buffer: 50 mM Tris·Cl (pH 7.5)/10 mM EDTA/0.3% (v/v) 2-mercaptoethanol (prepare fresh)
Lysis solution: 2 to 5 mg/ml Zymolyase 100T/rescue buffer *or* 100,000 U/ml β-glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma) diluted 1:50 in rescue buffer
10% (w/v) SDS
7.5 M ammonium acetate
Isopropanol
70% ethanol
TE buffer, pH 8.0 (*APPENDIX 2E*)

24-well microtiter plates
Centrifuge with microplate holders, refrigerated
Repeating micropipettor
37°C rotary shaker

Grow yeast cultures

1. Aliquot 2 ml of 2× Glu/CM –Trp medium into each well of a 24-well microtiter plate. Into each well, pick a putative positive colony. Grow overnight with shaking at 30°C.

The 2× minimal medium is used to maximize the yield of yeast. Four plates can generally be handled conveniently at once, based on the number that can be centrifuged simultaneously.

2. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant with a snap and return the plate to upright.
3. Swirl or lightly vortex the plate to resuspend cell pellets in remaining liquid. Add 1 ml water to each well and swirl lightly.

Cell pellets can most easily be resuspended in residual liquid before adding new solutions. Addition of liquid can be accomplished using a repeating pipettor.

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4. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant and resuspend pellet. Add 1 ml rescue buffer.
5. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant and resuspend pellet in the small volume of liquid remaining in the plate.

Lyse cells

6. To each well, add 25 µl lysis solution. Swirl or vortex to mix. Incubate (with cover on) on a rotary shaker ~1 hr at 37°C.

   Lysis solution need not be completely dissolved before use. By 1 hr, lysis should be obvious as coagulation of yeast into a white precipitate.

   Susceptibility of yeast strains to lytic enzymes varies. If lysis occurs rapidly, then less lytic enzyme should be used. If the lysis step is allowed to go too far, too much of the partially dissolved cell wall may contaminate the final material. Lysis can be judged by examining cells with a phase-contrast microscope. Living cells are white with a dark halo and dead cells are uniformly gray. Lysis leads to release of granular cell contents into the medium. Once cells are mostly gray and many are disrupted, much of the plasmid should have been released.

7. To each well, add 25 µl of 10% SDS. Mix gently by swirling to completely disperse the precipitates. Allow plates to sit 1 min at room temperature.

   At this point, the wells should contain a clear, somewhat viscous solution.

Purify plasmid

8. To each well, add 100 µl of 7.5 M ammonium acetate. Swirl gently, then incubate 15 min at –70°C or –20°C until frozen.

   Addition of acetate should result in the formation of a massive white precipitate of cell debris and SDS. The freezing step appears to improve removal of inhibitors of E. coli transformation.

9. Remove plate from freezer. Once it begins to thaw, centrifuge 15 min at 3000 × *g*, 4°C. Transfer 100 to 150 µl of the resulting clear supernatants to clean 24-well plates.

   In general, some contamination of the supernatant with pelleted material cannot be avoided. However, it is better to sacrifice yield in order to maintain purity.

10. To each well, add ~0.7 vol isopropanol. Mix by swirling and allow to precipitate 2 min at room temperature.

    A cloudy fine precipitate should form immediately after isopropanol is added.

11. Centrifuge 15 min at 3000 × *g*, 4°C. Shake off supernatant with a snap.
12. To each well, add ~1 ml cold 70% ethanol. Mix by swirling, centrifuge 5 min at 3000 × *g*, 4°C. Shake off supernatant with a snap, invert plates and blot well onto paper towel. Allow plates to air dry.
13. To each well, add 100 µl TE buffer. Swirl well and allow to rest on bench several minutes, until the pellets appear fully dissolved. Transfer preps to microcentrifuge tubes or 96-well plates for storage at –20°C.

    One to five microliters of each of the resulting preparations can be used to transform competent E. coli: for KC8, electroporation should be used (see Basic Protocol 2, step 21). Sometimes, the yield of transformants is low if E. coli carrying plasmids are not permitted time to increase the plasmid copy number above a critical threshold before the cells are placed on selective medium. Allow plenty of time for cells to express antibiotic resistance or the TRP1 gene before plating.

    If insufficient numbers of colonies are obtained by this approach, the final plasmid preparation can be resuspended in 20 µl instead of 100 µl TE buffer to concentrate the DNA stock.

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ADDITIONAL SPECIFICITY SCREENING

The three test plasmids outlined (pSH18-34, pRFHM1, and pEG202; see Basic Protocol 2, step 24) represent a minimal test series. If other LexA-bait proteins that are related to the bait protein used in the initial library screen are available, substantial amounts of information can be gathered by additional specificity tests. For example, if the initial bait protein was LexA fused to the leucine zipper of c-Fos, specificity screening of interactor-hunt positives against the leucine zippers of c-Jun or GCN4 in addition to that of c-Fos might allow discrimination between proteins that are specific for fos versus those that generically associate with leucine zippers.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Glycerol solution

65% (v/v) glycerol, sterile
0.1 M $MgSO_4$
25 mM Tris·Cl, pH 8.0 (*APPENDIX 2E*)
Store up to 1 year at room temperature

Laemmli sample buffer, 2×

10% (v/v) 2-mercaptoethanol (2-ME)
6% (w/v) SDS
20% (v/v) glycerol
0.2 mg/ml bromphenol blue
0.025× Laemmli stacking buffer (see recipe; optional)
Store up to 2 months at room temperature

This reagent can conveniently be prepared 10 ml at a time.

Laemmli stacking buffer, 2.5×

0.3 M Tris·Cl, pH 6.8
0.25% (w/v) SDS
Store up to 1 month at 4°C

Lysis solution

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)
10 mM EDTA
0.3% (v/v) 2-mercaptoethanol (2-ME), added just before use
2% (v/v) β-glucuronidase from *Helix pomatia* (Type HP-2; Sigma), added just before use

COMMENTARY

Background Information

Interaction-based cloning is derived from three experimental observations. In the first, Brent and Ptashne (1985) demonstrated that it was possible to assemble a novel, functional transcriptional activator by fusing the DNA-binding domain from one protein, LexA, to the activation domain from a second protein, GAL4. This allowed the use of a single reporter system containing a single DNA-binding motif, the *LexA* operator, to study transcriptional activation by any protein of interest. In the second, Ma and Ptashne (1988) built on this work to demonstrate that the activation domain could be brought to DNA by interaction with a DNA-binding domain. In the third, Fields and Song (1989), working independently of Ma and Ptashne, used two yeast proteins, SNF1 and SNF4, to make an SNF1 fusion to the DNA-binding domain of GAL4 and an SNF4 fusion to the GAL4 activation domain. They demonstrated that the strength of the SNF1-SNF4

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interaction was sufficient to allow activation through a GAL4 DNA-binding site. From this, they suggested the feasibility of selecting interacting proteins by performing screens of cDNA libraries made so that library-encoded proteins carried activating domains.

Several groups have developed cDNA library strategies along these lines, with some systems using LexA and others using GAL4 as the DNA-binding domain (Table 19.2.4). LexA and GAL4 each have different properties that should be considered when selecting a system. LexA is derived from a heterologous organism, has no known effect on the growth of yeast, possesses no residual transcriptional activity, can be used in GAL4$^+$ yeast, and can be used with a Gal-inducible promoter. Because GAL4 is an important yeast transcriptional activator, it has the disadvantage that experiments must be performed in gal4$^-$ yeast strains to avoid background due to activation of the reporter system by endogenous GAL4. Such gal4$^-$ strains are frequently less healthy and more difficult to transform than wild-type strains, and either libraries must be constitutively expressed or alternate inducible systems must be used. By contrast, the GAL4 DNA-binding domain may be more efficiently localized to the nucleus and may be preferred for some proteins (for a review of GAL4-based systems, see Bartel et al., 1993). Whichever system is used, it is important to remember that the bait protein constitutes a novel fusion protein whose properties may not exactly parallel those of the original unfused protein of interest. Although systems using the two-hybrid paradigm have been developed in mammalian cells (see Table 19.2.4), these have not been used effectively in library screens. It seems likely that the organism of choice for two-hybrid identification of novel partner proteins will remain yeast.

cDNAs that pass specificity tests are referred to as positives, or "true positives." In interactor hunts conducted to date, anywhere from zero to practically all isolated plasmids passed the final specificity test. If no positives are obtained, the tissue source for the library originally used may not be appropriate, and a different library may produce better results. However, there are some proteins for which no positives are found. Various explanations for this are provided below. Conversely, some library-encoded proteins are known to be isolated repeatedly using a series of unrelated baits, and these proteins demonstrate at least some specificity. One of these, heat shock protein 70, might be explained by positing that it assists the folding of some LexA-fused bait proteins, or alternatively, that these bait proteins are not normally folded. This example illustrates the point that the physiological relevance of even quite specific interactions may sometimes be obscure.

Because the screen involves plating multiple cells to Gal/CM –Ura, –His, –Trp, –Leu dropout medium for each primary transformant obtained, multiple reisolates of true positive cDNAs are frequently obtained. If a large number of specific positives are obtained, it is generally a good idea to attempt to sort them into classes—for example, digesting minipreps of positives with *Eco*RI, *Xho*I, and *Hae*III will generate a fingerprint of sufficient resolution to determine whether multiple reisolates of a small number of clones or single isolates of many different clones have been obtained. The former situation is a good indication that the system is working well.

An important issue that arises in an interactor hunt is the question of how biologically relevant interacting proteins that are isolated are likely to be. This leads directly to the question of what K_d of association two molecules must have to be detected by an interactor hunt. In fact, this is not at all a simple issue. For the system described here, most fusion proteins appear to be expressed at levels ranging from 50 nM to 1 μM (Golemis and Brent, 1992). Given the strength of the *GAL* promoter, it is likely that many library-encoded proteins are expressed at similarly high levels, ≥1 μM in the nucleus (Golemis and Brent, 1992). At this concentration, which is in considerable excess over the nuclear concentration of operator-bound bait protein, a cDNA library–encoded protein should half-maximally occupy the DNA-bound bait protein if it possesses a K_d of 10^{-6} M, making it theoretically possible that very-low-affinity interactions could be detected. Such interactions have been observed in some cases. In contrast, some interactions that have been previously established using other methods and are predicted by known K_d to be easily detected by these means, either are not detected or are detected only weakly (Finley and Brent, 1994; Estojak et al., 1995). Because of the conservation of many proteins between lower and higher eukaryotes, one explanation for this observation is that either one or both of the partners being tested is being sequestered from the desired interaction by fortuitous association with an endogenous yeast protein. A reasonably complete investigation of the degree of correlation between in vitro determina-

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tions of interaction affinity and apparent strength of interaction in the interaction trap is included in Estojak et al. (1995). The result of this investigation suggests it is important to measure the affinity of detected interactions under different conditions, using a second assay system, rather than to draw conclusions about affinity based on detection in the interaction trap.

A number of different plasmids can be used for conducting an interactor hunt. Their properties are summarized in Tables 19.2.1 and 19.2.2. Because of the generous and open scientific exchange between investigators using the system, the number of available plasmids and other components has greatly expanded since the appearance of the initial two-hybrid reagents, facilitating the study of proteins inaccessible by the original system.

The original parent plasmid for generating LexA fusions, pEG202 is a derivative of 202 + PL (Ruden et al., 1991; see Fig. 19.2.3) that contains an expanded polylinker region. The available cloning sites in pEG202 include *Eco*RI, *Bam*HI, *Sal*I, *Nco*I, *Not*I, and *Xho*I, with the reading frame as described in the legend to Figure 19.2.3. Since the original presentation of this system, a number of groups have developed variants of this plasmid that address specialized research needs. Those currently available, as well as purposes for which they are suited, are listed in Table 19.2.1. pGilda, created by David A. Shaywitz, places the LexA-fusion cassette under the control of the inducible *GAL1* promoter, allowing expression of the bait protein for limited times during library screening, reducing the exposure of yeast to toxic baits. pJK202, created by Joanne Kamens, adds nuclear localization sequences to pEG202, facilitating assay of the function of proteins lacking internal nuclear localization sequences. pNLexA, created by Ian York, places LexA carboxy-terminal in the fusion domain, allowing assay of interactions that require an unblocked amino-terminus on the bait protein. pEE202I, created by Mike Watson and Rich Buckholz, allows chromosomal integration of a pEG202-like bait, thus reducing expression levels so they are more physiological for bait proteins normally present at low levels intracellularly. All of these have been extensively tested by numerous researchers. pGilda, pJK202, and pEE202I work with complete reliability. pNLexA works effectively with ~50% of the fusion domains tried, but synthesizes only very low levels of protein (relative to expression of the same fusion domain as a pEG202 fusion) with the remaining 50%. Attachment of fusion domains amino-terminal either to *LexA* or *GAL4* has been generally problematic in the hands of many investigators; it may be that appending additional protein sequences to the amino termini of these proteins is destabilizing, although the problem has not been rigorously investigated.

A series of *lacZ* reporters of differing sensitivity to transcriptional activation can be used to detect interactions of varying affinity (see Table 19.2.2). These plasmids are *LexA* operator–containing derivatives of the plasmid LR1Δ1 (West et al., 1984). In LR1Δ1, a minimal *GAL1* promoter lacking the *GAL1* upstream activating sequences (GAL_{UAS}) is located upstream of the bacterial *lacZ* gene. In pSH18-34, eight *LexA* operators have been cloned into an *Xho*I site located 167 bp upstream of the *lacZ* gene (S. Hanes, unpub. observ.). pJK103 and pRB1840 contain two and one operator, respectively.

pJK101 is similar to pSH18-34, except that it contains the *GAL1* upstream activating sequences (GAL_{UAS}) upstream of two *LexA* operator sites. A derivative of del20B (West et al., 1984), it is used in the repression assay (Brent and Ptashne, 1984; see Fig. 19.2.5) to assess LexA fusion binding to operator.

pSH17-4 is a *HIS3* 2μm plasmid that encodes LexA fused to the activation domain of the yeast activator GAL4. EGY48 cells bearing this plasmid will produce colonies in overnight growth on medium lacking Leu, and yeast that additionally contain pSH18-34 will turn deep blue on plates containing Xgal. This plasmid serves as a positive control for the activation of transcription.

pRFHM1 is a *HIS3* 2μm plasmid that encodes LexA fused to the N-terminus of the *Drosophila* protein bicoid. The plasmid has no ability to activate transcription, so EGY48 cells that contain pRFHM1 and pSH18-34 do not grow on –Leu medium and remain white on plates containing Xgal. pRFHM1 is a good control for specificity testing, because it has been demonstrated to be sticky—that is, to associate with a number of library-encoded proteins that are clearly nonphysiological interactors (R. Finley, Wayne State University, Detroit, Mich., unpub. observ.).

This protocol uses interaction libraries (Table 19.2.3) made in pJG4-5 or its derivatives (see Fig. 19.2.6). pJG4-5 was developed to facilitate isolation and characterization of novel proteins in interactor hunts (Gyuris et al., 1993). The pJG4-5 cDNA library expression

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cassette is under control of the *GAL1* promoter, so library proteins are expressed in the presence of galactose (Gal) but not glucose (Glu). This conditional expression has a number of advantages, the most important of which is that many false-positives obtained in screens can be easily eliminated because they do not demonstrate a Gal-dependent phenotype. The expression cassette consists of an ATG to start translation, a nuclear localization signal to extend the interaction trap's range to include proteins that are normally predominantly localized in the cytoplasm, an activation domain (acid blob; Ma and Ptashne, 1987), the hemagglutinin epitope tag to permit rapid assessment of the size of encoded proteins, *Eco*RI-*Xho*I sites designed to receive directionally synthesized cDNAs, and the alcohol dehydrogenase (*ADH*) termination sequences to enhance the production of high levels of library protein. The plasmid also contains the *TRP1* auxotrophy marker and 2μm origin for propagation in yeast. A derivative plasmid, pJG4-5I, was created by Mike Watson and Richard Buckholz to facilitate chromosomal integration of the activation domain fusion expression plasmid.

A series of recently developed derivatives of pEG202, pJG4-5, and *lacZ* reporter plasmids (MW101 to MW112) alter the antibiotic resistance markers on these plasmids from ampicillin (Ap^r) to either kanamycin (Km^r) or chloramphenicol (Cm^r; Watson et al., 1996). Judiciously mixing and matching these plasmids in conjunction with Ap^r libraries would considerably reduce work subsequent to library screening, because the KC8 transformation, which involves *trpC* complementation in bacteria, could be omitted.

EGY48 and EGY191 (see Table 19.2.2) are both derivatives of the strain U457 (a gift of Rodney Rothstein, Columbia University, New York, N.Y.) in which the endogenous *LEU2* gene has been replaced by homologous recombination with *LEU2* reporters carrying varying numbers of *LexA* operators, using a procedure detailed in Estojak et al. (1995).

Interaction Trap–compatible reagents have recently become commercially available; Clontech and Invitrogen were the first to market such reagents and have recently been joined by OriGene. All suppliers use systems with the most sensitive reporters (EGY48 and pSH18-34), and provide their own positive and negative controls for testing activation or interaction between defined proteins. For expression of bait and library proteins, the Clontech Matchmaker LexA two-hybrid system and the OriGene Duplex-A system use some of the basic set of plasmids described here (see Table 19.2.1 for availability). Forward sequencing primers for bait and library plasmids are included in the Clontech kit, and Insert Screening Amplimer Sets for both plasmids can be acquired separately. Additional related products from Clontech include KC8 competent cells, anti-LexA monoclonal antibodies, a yeast transformation system, a yeast plasmid isolation kit, and an EGY48 partner strain for yeast mating to facilitate the analysis of interaction specificity. OriGene has a generally similar product line to Clontech. In contrast, Invitrogen has substantially modified the Interaction Trap core reagents to develop its own bait and library plasmids. pHybLex/Zeo, a novel bait plasmid, is ~50% smaller than the original pEG202 (making it easier to clone into), and it has an enriched polylinker. Significantly, it replaces both the Ap^r and *HIS3* genes with a novel gene that confers resistance to the antibiotic Zeocin (supplied with the kit), which provides selection in both bacteria and yeast. This elimination of auxotrophic selection for the bait plasmid renders the LexA-fusion construct usable with libraries and strains from all existing two-hybrid systems and additionally facilitates the direct selection of library plasmid in strains other than KC8. Some changes, which are designed to make the vector easier to use, have also been introduced in the library vector pYESTrp (e.g., it uses a V5 epitope tag for protein detection). The Invitrogen kit, termed Hybrid Hunter, includes the bait/library/reporter plasmids and EGY48 yeast strain as noted, and additionally includes primer sets for bait and library plasmids and the L40 yeast strain, should an investigator wish to use a *HIS3* auxotrophy selection. Additional related products from Invitrogen include antibodies for detection of bait and prey fusion proteins (anti-LexA and anti-V5), pJG4-5 library vector primers, and a Transformation Kit.

A significant advantage of the entry of commercial entities into the Interaction Trap field is the rapid increase in the number of compatible cDNA libraries. A list of currently available premade libraries available from these companies is presented in Table 19.2.3, and custom-made libraries are also available upon request. Because new libraries and other related reagents are being constantly added to the line of two-hybrid related products, it is advisable to contact the companies or visit their Web sites (*www.clontech.com, www.invitrogen.com,* and *www.origene.com*) for the latest information.

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Finally, over the last several years, a number of groups have adapted basic two-hybrid strategies to more specialized applications, and they have devised strategies to broaden their basic functionality. Interaction Mating (Finley and Brent, 1994) has been used to establish extended networks of targeted protein-protein interaction. In this approach, a panel of LexA-fused proteins are transformed into a *MATa* haploid selective strain (such as RFY206), a panel of activation-domain fused proteins are transformed into a suitable MATα haploid (such as EG448), and the two panels are cross-gridded against each other for mating. Selected diploids are then screened by replica plating to selective medium. This approach complements library screening in large-scale applications, such as proposed definition of interaction maps for entire genomes (Bartel et al., 1996).

Interaction mating has also provided the basis for an alternative two-hybrid hunt protocol (see Alternate Protocol 2), useful in cases when a single library will be screened with different baits. In this approach (Bendixen et al., 1994; Finley and Brent, 1994: Kolonin and Finley, 1998), a library is introduced into a single strain, like EGY48, and aliquots are stored frozen. To conduct a hunt, an aliquot is thawed and mated with a strain expressing a bait. This allows one to avoid repeated high-efficiency transformations, since a single library transformation can provide enough pretransformed yeast to conduct dozens of interactor hunts. Moreover, some yeast strains pretransformed with libraries are becoming commercially available, which may eliminate altogether the need to conduct a high-efficiency library transformation for some researchers.

Two-hybrid approaches have been shown to be effective in identifying small peptides with biological activities on selected baits (Yang et al., 1995; Colas et al., 1996), which may prove to be useful as a guide to targeted drug design. Rapid screening protocols have been devised using custom-synthesized libraries expressing sheared plasmid DNA to facilitate rapid mapping of interaction interfaces (Stagljar et al., 1996). Osborne and coworkers have demonstrated the effectiveness of a tribrid (or tri-hybrid) approach, in which an additional plasmid expresses a tyrosine kinase to specifically modify a bait protein, allowing detection of SH2-domain-containing partner proteins that recognize specific phosphotyrosine residues (Osborne et al., 1995). A variety of more elaborate tribrid approaches, in which a DNA-binding domain fused protein is used to present an intermediate nonprotein compound for interaction with a library, have been developed and proven effective. These approaches have allowed the identification of proteins binding specific drug ligands (Chiu et al., 1994; Licitra and Liu, 1996), as well as the identification of proteins binding to RNA sequences (SenGupta et al., 1996; Wang et al., 1996). It is expected that the range of utility of these systems will continue to expand.

Critical Parameters and Troubleshooting

To maximize chances of a successful interactor hunt, a number of parameters should be taken into account. Before attempting a screen, bait proteins should be carefully tested to ensure that they have little or no intrinsic ability to activate transcription. Bait proteins must be expressed at reasonably high levels and must be able to enter the yeast nucleus and bind DNA (as confirmed by the repression assay). Optimally, integrity and levels of bait proteins should be confirmed by immunoblot analysis, using an antibody to either LexA or the fused domain. In particular, at this time, bait proteins that have extensive transmembrane domains or are normally excluded from the nucleus are not likely to be productively used in a library screen. Proteins that are moderate to strong activators will need to be truncated to remove activating domains before they can be used.

If a protein neither activates nor represses, the most likely reason is that it is not being made. This can be determined by immunoblot analysis of a crude lysate protein extract of EGY48 (*UNIT 10.10*; Samson et al., 1989) containing the plasmid, using anti-LexA antibodies as primary antiserum. If the full protein is not made, it may be possible to express truncated derivatives of the protein. If the protein is made, but still does not repress, it may not enter the yeast nucleus effectively, although this appears to be a relatively rare problem. In this case, introducing the coding sequence for the fused moieity into a LexA fusion vector containing a nuclear localization motif (e.g., pJK202; J. Kamens, BASF, Worcester, Mass., unpub. observ.) may solve the problem.

The test for the leucine (Leu) requirement is extremely important to determine whether the bait protein is likely to yield an unworkably high background. The *LEU2* reporter in EGY48 is more sensitive than the pSH18-34 reporter for some baits (Estojak et al., 1995). Therefore, it is possible that a bait protein demonstrating little or no signal in a β-galac-

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tosidase assay may nevertheless permit some growth on –Leu medium. If this occurs, there are several options. First, a less sensitive strain can be used, as described in the text. Second, background can sometimes be reduced further by making the EGY strain diploid (e.g., D. Krainc, Harvard Medical School, Boston, Mass.; R. Finley and R. Brent, unpub. observ.) or by performing the hunt by interaction mating as described in Alternate Protocol 2. A third option is to attempt to truncate the bait protein to remove activating function. In general, it is useful to extrapolate from the number of cells that grow on –Leu medium to the number that would be obtained in an actual library screen, and determine if this is a background level that can be tolerated. For example, if two colonies arise from 100,000 plated cells on –Leu medium, 200 to 400 would be expected in an actual screen of 10^6 cDNAs. Although this is a high initial number of positives, the vast majority should be eliminated immediately through easily performed controls. This is a judgment call. Finally, very rarely it happens that a bait that appears to be well behaved and negative for transcriptional activation through all characterization steps will suddenly develop a very high background of transcriptional activation following library transformation. The reason for this is currently obscure, and no means of addressing this problem has as yet been found: such baits are hence inappropriate for use in screens.

The protocols described in this unit use initial screening with the most sensitive reporters followed by substitution with less sensitive reporters if activation is detected. An obvious question is, why not start out working with extremely stringent reporters and know immediately whether the system is workable? In fact, some researchers routinely use a combination of pJK103 or pRB1840 with EGY191, and obtain proteins that to date appear to be biologically relevant partners from library screens. However, extensive comparison studies using interactors of defined in vitro affinity with different combinations of *LacZ* and *LEU2* reporters (Estojak et al., 1995) have indicated that although the most sensitive reporters (pSH18-34) may in some cases be prone to background problems, the most stringent reporters (EGY191, pRB1840) may miss some interactions that certainly are biologically relevant and occur inside cells. In the end, the choice of reporters devolves to the preference of individual investigators: the bias of the authors is to cast a broad net in the early stages of a screen, and hence to use more sensitive reporters when practicable.

It is important to move expeditiously through characterization steps and to handle yeast transformed with bait plasmids with care. In cases where yeasts have been maintained on plates for extended periods (e.g., 4 days at room temperature or >2 to 3 weeks at 4°C), unexpected problems may crop up in subsequent library screens.

The transformation protocol is a version of the lithium acetate transformation protocol described by Schiestl and Gietz (1989) and Gietz et al. (1992; see *APPENDIX 4L*) that maximizes transformation efficiency in *Saccharomyces cerevisiae* and produces up to 10^5 colonies/μg plasmid DNA. In contrast to *Escherichia coli*, the maximum efficiency of transformation for *S. cerevisiae* is ~10^4 to 10^5/μg input DNA. It is extremely important to optimize transformation conditions before attempting an interactor hunt. Perform small-scale pilot transformations to ensure this efficiency is attained and to avoid having to use prohibitive quantities of library DNA. In addition, as for any effort of this type, it is a good idea to obtain or construct a library from a tissue source in which the bait protein is known to be biologically relevant.

In practice, the majority of proteins isolated by interaction with a LexA fusion turn out to be specific for the fused domain; a smaller number are nonspecifically sticky, and to date there appears to have been only one isolation from a eukaryotic library of a protein specific for LexA. However, it is generally informative to retest positive clones on more than one LexA bait protein; ideally, library-derived clones should be tested against the LexA fusion used for their isolation, several LexA fusions to proteins that are clearly unrelated to the original fusion, and if possible, several LexA fusions that there is reason to believe are related to the initial protein (e.g., if the initial probe was LexA-Fos, a good related set would include LexA-Jun and LexA-GCN4).

Colony selection for master plate production is one of the more variable parts of the procedure. For strong interactors, colonies will grow up in 2 days. However, if plates are left at 30°C, new colonies will continue to appear every day. Those that appear rapidly are most likely to reflect interactors that are biologically relevant to the bait protein. Those that appear later may or may not be relevant. However, many parameters can delay the time of colony formation of cells that contain valid interactions, including the strength of the interaction

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and the level of expression of the library-encoded protein.

Anticipated Results

Depending on the protein used as bait, anywhere from zero to hundreds of specific interactors will be obtained from 10^6 primary transformants.

Time Considerations

If all goes well, once the required constructions have been made it will take ~1 week to perform yeast transformations, obtain colonies, and determine whether bait proteins are appropriate. It will take a second week to perform library transformations, replate to selective medium, and obtain putative positives. A third week will be required to rescue the plasmid from the yeast, passage it through *E. coli*, transform fresh yeast, and confirm specificity.

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Internet Resources

http://www.clontech.com

http://cmmg.biosci.wayne.edu/rfinley/lab.html

Source of two-hybrid information, protocols, and links.

http://www.invitrogen.com

http://www.origene.com

Commercial sources for basic plasmids, strains, and libraries for interaction trap experiments.

brent@molsci.org

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Sources of interaction trap plasmids for specialized interactions.

http://www.fccc.edu:80/research/labs/golemis/InteractionTrapInWork/html

Database for false postive proteins detected in interaction trap experiments; analysis of two-hybrid usage.

http://xanadu.mgh.harvard.edu/brentlabhome page4.html

Database of interaction trap protocols and related issues.

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Growth and Manipulation of Yeast

PREPARATION OF SELECTED YEAST MEDIA

Like *Escherichia coli*, yeast can be grown in either liquid media or on the surface of (or embedded in) solid agar plates. Yeast cells grow well on a minimal medium containing dextrose (glucose) as a carbon source and salts which supply nitrogen, phosphorus, and trace metals. Yeast cells grow much more rapidly, however, in the presence of protein and yeast cell extract hydrolysates, which provide amino acids, nucleotide precursors, vitamins, and other metabolites which the cells would normally synthesize de novo. During exponential or log-phase growth, yeast cells divide every 90 min when grown in such media.

Preparation of sterile media of consistently high quality is essential for the genetic manipulation of yeast. Autoclaving is usually carried out for 15 min at 15 lb/in^2, but times should be increased when large amounts of media are being prepared (20 min for 4 to 6 liters and 25 min for 6 to 12 liters).

Liquid Media

Ingredients for liquid media are dissolved in water to 1 liter, mixed until completely dissolved, and autoclaved in 100- or 500-ml media bottles. Alternatively, liquid media can be filter sterilized, resulting in faster preparation, less carmelization (of dextrose), and faster growth of cells. Recipes for "premixes" are provided to minimize the number of materials that must be weighed each time medium is prepared. When preparing premixes, break up any large chunks of dextrose before mixing with other components, then shake the container vigorously until contents are homogenized. It is convenient to make the premix in the empty plastic containers in which 2.5 kg of dextrose is packaged. Throughout this chapter, YNB –AA/AS refers to yeast nitrogen base without amino acids or ammonium sulfate. (See listing of recommended suppliers of ingredients in unit introduction above.)

Rich medium

YPD medium (YEPD medium)

Per liter:	*Premix:*	*Final concentration:*
10 g yeast extract	250 g yeast extract	1% yeast extract
20 g peptone	500 g peptone	2% peptone
20 g dextrose	500 g dextrose	2% dextrose
	Use 50 g/liter	

It is preferable to use a 20% (10×) solution of dextrose that has been filter sterilized or autoclaved separately (and added to the other ingredients after autoclaving) to prevent darkening of the media and to promote optimal growth.

Minimal media

Minimal medium [synthetic dextrose (SD) medium]

Per liter:	*Premix:*	*Final concentration:*
1.7 g YNB –AA/AS	68 g YNB –AA/AS	0.17% YNB –AA/AS
5 g $(NH_4)_2SO_4$	200 g $(NH_4)_2SO_4$	0.5% $(NH_4)_2SO_4$
20 g dextrose	800 g dextrose	2% dextrose
	Use 27 g/liter	

Contributed by Douglas A. Treco, Ann Reynolds, and Victoria Lundblad

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This minimal medium can support the growth of yeast which have no nutritional requirements. However, it is used most often as a basal medium to which other supplements are added (see CM dropout medium below).

Complete minimal (CM) dropout medium, per liter

1.3 g dropout powder (Table A.4L.1)
1.7 g YNB –AA/AS
5 g $(NH_4)_2SO_4$
20 g dextrose
Alternatively, replace last three ingredients with 27 g minimal (SD) medium premix.

CM dropout powder, also known as minus or omission powder, lacks a single nutrient but contains the other nutrients listed in Table A.4L.1. Complete minimal (CM) dropout medium is used to test for genes involved in biosynthetic pathways and to select for gene function in transformation experiments. To test for a gene involved in histidine biosynthesis, determine if the yeast strain in question can grow on CM minus histidine (–His) or "histidine dropout" plates. It is convenient to make several dropout powders, each lacking a single nutrient, to avoid weighing each component separately for all the different dropout plates required in the laboratory.

It may be preferable to use a 10× solution of dropout powder (i.e., 13 g of dropout powder in 100 ml water) that has been "sterilized" separately (and added to the other ingredients after autoclaving) to improve the growth rate in this medium.

Table A4.L.1 Nutrient Concentrations for Dropout Powders[a]

Nutrient[b]	Amount in dropout powder (g)[c]	Final conc. in prepared media (μg/ml)	Liquid stock conc. (mg/100 ml)[d]
Adenine (hemisulfate salt)	2.5	40	500
L-arginine (HCl)	1.2	20	240
L-aspartic acid[e]	6.0	100	1200
L-glutamic acid (monosodium salt)	6.0	100	1200
L-histidine	1.2	20	240
L-leucine[f]	3.6	60	720
L-lysine (mono-HCl)	1.8	30	360
L-methionine	1.2	20	240
L-phenylalanine	3.0	50	600
L-serine	22.5	375	4500
L-threonine[e]	12.0	200	2400
L-tryptophan	2.4	40	480
L-tyrosine	1.8	30	180[f]
L-valine	9.0	150	1800
Uracil	1.2	20	240

[a]CM dropout powder lacks a single nutrient but contains the other nutrients listed in this table. Nomenclature in this manual refers to, e.g., a preparation that omits histidine as histidine dropout powder or CM medium –His. Conditions from Sherman et al. (1979).

[b]Amino acids not listed here can be added to a final concentration of 40 μg/ml (40 mg/liter).

[c]Grind powders into a homogeneous mixture with a clean, dry mortar and pestle. Store in a clean, dry bottle or a covered flask.

[d]Use 8.3 ml/liter of each stock for special nutritional requirements. Store adenine, aspartic acid, glutamic acid, leucine, phenylalanine, tyrosine, and uracil solutions at room temperature. All others should be stored at 4°C.

[e]Although these amino acids can be used reliably when included in autoclaved media, they supplement growth better when added after autoclaving.

[f]Use 16.6 ml/liter for L-tyrosine nutritional requirement.

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Solid Media

For all plates, agar is added at a concentration of 2% (20 g/liter). A pellet of sodium hydroxide (~0.1 g) should be added per liter to raise the pH enough to prevent agar breakdown during autoclaving. In addition, add a stir bar to facilitate mixing after autoclaving. After autoclaving, flasks are left for 45 to 60 min at room temperature until cooled to 50° to 60°C. (Drugs and other nutrients are added after 30 min at room temperature.) Just prior to pouring, put the flask on a stir plate at medium to high speed and mix until contents are homogeneous (~5 min). After pouring, a few bubbles can be removed from the agar surface by passing the flame of a Bunsen burner lightly over the surface of the molten agar ("flaming" the plates). One liter of medium will yield 30 to 35 plates.

Most plates can be stored at room temperature for ≤4 months. Plates containing drugs (cycloheximide, 5-fluoroorotic, canavanine, and L-α-aminoadipic acid) or 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Xgal; 20 μg/ml; *APPENDIX 4A*) are stable for 2 to 3 months when stored at 4°C.

Minimal plates and rich plates

YPD, minimal, and CM dropout plates

Per liter: Follow recipes for liquid medium above, adding 20 g agar and a pellet of NaOH.

Premixes: Follow recipes for liquid medium premixes above, adding 500 g agar for YPD premix and 800 g agar for minimal premix. To prepare plates, add one NaOH pellet and the following amounts of premix (per liter):

YPD plates—70 g YPD plate premix
Minimal plates—47 g minimal plate premix
CM dropout plates—47 g minimal plate premix + 1.3 g dropout powder

Specialty plates

Xgal plates, per liter

1.7 g YNB –AA/AS
5 g $(NH_4)_2SO_4$
20 g dextrose
20 g agar
0.8 g dropout powder (omitting appropriate amino acids; see Table A4.L.1)
NaOH pellet
(Alternatively, replace first four ingredients with 47 g minimal plate premix.)

Add H_2O to 900 ml and autoclave. Add 100 ml of 0.7 M potassium phosphate, pH 7.0, and 2 ml of 20 mg/ml Xgal prepared in 100% *N,N*-dimethylformamide (stored as frozen stock).

STRAIN STORAGE AND REVIVAL

Yeast strains can be stored at –70°C in 15% glycerol (viable for >5 years), or at 4°C on slants consisting of rich medium supplemented with potato starch (viable for 1 to 2 years).

Preparation and Inoculation of Frozen Stocks

Make a solution of 30% (w/v) glycerol. Pipet 1 ml into 15 × 45–mm, 4-ml screw-cap vials. Loosely cap the vials and autoclave 15 min.

To inoculate vials for storage, add 1 ml of a late-log or early-stationary phase culture, mix, and set on dry ice. Store at –70°C. Revive by scraping some of the cells off the frozen

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surface and streak onto plates. Do not thaw the entire vial. Cells can also be stored in the same way by adding 80 µl dimethyl sulfoxide (DMSO) to 1 ml cells (8% v/v) and storing at –70°C.

BASIC PROTOCOL 1

ASSAY FOR β-GALACTOSIDASE IN LIQUID CULTURES

Materials

YPD medium (see recipe under Liquid Media)
Z buffer (see recipe in Reagents and Solutions)
0.1% sodium dodecyl sulfate (SDS)
Chloroform
4 mg/ml *O*-nitrophenyl-β-D-galactoside (ONPG) in 0.1 M potassium phosphate, pH 7.0 (see *APPENDIX 2E* for buffer; filter sterilize and store frozen)
1 M Na_2CO_3
30°C water bath

1. Inoculate 5 ml YPD (or appropriate) medium with single yeast colony. Grow 2 to 3 independent single-colony cultures of a strain containing *lacZ* fusion protein overnight at 30°C. If fusion is on a plasmid, grow cells in medium that selects for plasmid.
2. Inoculate 5 ml YPD medium (or appropriate selective medium and/or inducing conditions) with 20 to 50 µl of each overnight culture. Grow to mid- or late-log phase: 0.5–1 × 10^8 cells/ml (OD_{600} = 2.0) for rich medium or 2–5 × 10^7 cells/ml (OD_{600} = 0.5 to 1.0) for minimal medium.

   If goal is to investigate activity of yeast promoter under conditions that induce its expression, grow cells in parallel under inducing and noninducing conditions.

3. Centrifuge cells 5 min at 1100 × *g*. Resuspend in equal volume of Z buffer and place on ice. Determine OD_{600} for each sample.

   If anticipated level of β-galactosidase activity is low, it may be necessary to concentrate cells. For cells demonstrating 100 to 1000 U of activity within 30 min to 4 hr, concentration is not necessary.

4. Set up the following two reaction tubes for each sample (1 ml each), with mixing: (a) 100 µl cells with 900 µl Z buffer and (b) 50 µl cells with 950 µl Z buffer.
5. Add 1 drop of 0.1% SDS and 2 drops chloroform to each sample using a Pasteur pipet. Vortex 10 to 15 sec and equilibrate 15 min in 30°C water bath.
6. Add 0.2 ml of 4 mg/ml ONPG; vortex 5 sec. Place in 30°C water bath and begin timing. When medium-yellow color (OD_{420} = 0.3 to 0.7) has developed, add 0.5 ml of 1 M Na_2CO_3 and note time.
7. Centrifuge cells 5 min at 1100 × *g*. Determine OD_{420} and OD_{550} of supernatant.

   If the cell debris has been well pelleted, the OD_{550} is usually zero and therefore is not necessary to read.

8. Calculate units with the following equation:

$$U = \frac{1000 \times [(OD_{420}) - (1.75 \times OD_{550})]}{(t) \times (v) \times (OD_{600})}$$

where t = time of reaction (min); v = volume of culture used in assay (ml); OD_{600} = cell density at the start of the assay; OD_{420} = combination of absorbance by *o*-ni-

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trophenol and light scattering by cell debris; and OD_{550} = light scattering by cell debris.

TRANSFORMATION USING LITHIUM ACETATE

BASIC PROTOCOL 2

This is the most commonly used method for yeast transformation. It is reasonably fast and provides transformation efficiencies of 10^5 to 10^6 transformations/µg.

NOTE: All solutions and glassware coming into contact with yeast cells must be sterile. Traces of soap on glassware may decrease the transformation efficiency. In addition, the water used for washes and in solution preparation must be of the highest quality.

Materials

YPD medium (see recipe under Liquid Media)
Yeast strain to be transformed
YPAD medium: YPD medium supplemented with 30 mg/liter adenine hemisulfate
Highest-quality sterile H_2O
10× TE buffer, pH 7.5 (see *APPENDIX 2E* for 1× recipe), sterile
10× lithium acetate stock solution: 1 M lithium acetate, pH 7.5 (adjust pH with dilute acetic acid), filter sterilized
DNA: high-molecular-weight, single-stranded carrier DNA and transforming DNA
50% (w/v) polyethylene glycol (PEG 4000 or 3350 (do not use PEG 8000), filter sterilized
CM dropout plates (see recipe under Solid Media) prepared with Difco agar

30°C incubator with shaker
Sorvall GSA and SS-34 rotors (or equivalents)
42°C water bath

1. Two days before the experiment, inoculate 5 ml YPD medium with a single yeast colony of the strain to be transformed. Grow overnight to saturation at 30°C.
2. The night before transformation, inoculate a 1-liter sterile flask containing 300 ml YPAD medium with an appropriate amount of the saturated culture and grow overnight at 30°C to 1×10^7 cells/ml ($OD_{600} \cong 0.3$ to 0.5, depending on strain). For 2- to 3-fold higher efficiency, dilute at this point to 2×10^6 cells/ml in fresh YPAD medium and grow for another 1 to 2 generations (2 to 4 hr).

   Because growth phase and cell density are important in achieving the highest transformation efficiency, the easiest approach is to inoculate three independent flasks with varying amounts of the saturated overnight culture (try 1, 5, and 25 µl for a 12-hr growth period), then check the OD_{600} of each flask the next day.

3. Harvest cells by centrifuging 5 min at 4000 × *g*, room temperature. Resuspend in 10 ml highest-quality sterile water. Transfer to a smaller centrifuge tube and pellet cells by centrifuging 5 min at 5000 to 6000 × *g*, room temperature.
4. Resuspend in 1.5 ml buffered lithium solution, freshly prepared as follows:

   1 vol 10× TE buffer, pH 7.5
   1 vol 10× lithium acetate stock solution
   8 vol sterile water.

5. For each transformation, mix 200 µg carrier DNA with ≤5 µg transforming DNA in a sterile 1.5-ml microcentrifuge tube. Keep total volume of DNA ≤20 µl.

   Maximal transformation efficiency is achieved by repeating the denaturation cycle (boiling and chilling) of carrier DNA immediately prior to use.

6. Add 200 µl yeast suspension to each microcentrifuge tube. Add 1.2 ml PEG solution, freshly prepared as follows:

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8 vol 50% PEG
1 vol 10× TE buffer, pH 7.5
1 vol 10× lithium acetate stock solution.

Shake 30 min at 30°C.

7. Heat shock exactly 15 min at 42°C. Microcentrifuge 5 sec at room temperature. Resuspend yeast in 200 µl to 1 ml of 1× TE buffer (freshly prepared from 10× stock) and spread up to 200 µl onto CM dropout plates made with Difco agar. Incubate 2 to 5 days at 30°C until transformants appear.

REAGENTS AND SOLUTIONS

Use Milli-Q water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Z buffer

16.1 g $Na_2HPO_4 \cdot 7H_2O$ (60 mM final)
5.5 g $NaH_2PO_4 \cdot H_2O$ (40 mM final)
0.75 g KC1 (10 mM final)
0.246 g $MgSO_4 \cdot 7H_2O$ (1 mM final)
2.7 ml 2-mercaptoethanol (50 mM final)
Adjust to pH 7.0 and bring to 1 liter with H_2O
Do not autoclave

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SUPPLEMENT INDEX

How to Use This Index

A new main index is provided once a year. Thereafter, with the next three supplements, a cumulative supplement index, which amends the main index, will be provided. Thus, readers should consult both the main index and the supplement index when seeking an entry, as page numbers may change for replacement units and new entries will added.

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Current Protocols in Protein Science

Supplement 15 **New and Revised Topics** **March 1999**

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☑ Ion-Exchange Chromatography *(New)* UNIT 8.2

☑ Enzymatic Approaches for Obtaining Amino Acid Sequence: On-Target Ladder Sequencing *(New)* UNIT 16.7

☑ Native Chemical Ligation of Polypeptides *(New)* UNIT 18.4

☑ Phage-Based Expression Cloning to Identify Interacting Proteins *(New)* UNIT 19.3

Coming soon ...

☑ Characterizing Recombinant Proteins Using Gel-Filtration HPLC and Mass Spectrometry *(New)*

☑ Peptide Demdrimers as Protein Mimetics *(New)*

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Sequence Similarity Searching Using the BLAST Family of Programs

Database sequence similarity searching is carried out thousands of times each day by researchers worldwide. Scientists in traditional laboratories use search results, for example, to infer the functions of newly discovered cDNAs, predict new members of gene families, and explore evolutionary relationships between sequences. In turn, they populate sequence databases with biochemically and/or genetically characterized sequences. With the advent of whole-genome sequencing, a new breed of scientist is now using these characterized sequences to predict the location and function of coding and regulatory regions in large segments of genomic DNA. Their contribution to the sequence databases is the submission, at a rapid pace, of generally uncharacterized mRNA and genomic DNA sequences. These data can then be used by the first group of scientists to enhance the understanding of their sequences. Both types of sequencing efforts have greatly increased the size and quality of the sequence databases in recent years. Thus, sequence similarity searching has become a valuable research tool for all molecular biologists (Altschul et al., 1994; Schuler, 1998; also see *UNIT 2.1*).

Over the years, a number of algorithms have been implemented that allow searching of sequence databases. The most useful of these tools should share the following characteristics: (1) Speed. Because today's databases are so large, the programs must be fast in order to process megabases of sequence in seconds. (2) Sensitivity. The programs must report all potentially interesting similarities. (3) Rigorous statistics. The programs must provide a way to evaluate the significance of the results. (4) Ease of use. Scientists with no formal training in sequence-analysis algorithms should understand how to use the programs and interpret the results. Advanced users should have the option to tailor the programs to their needs. (5) Access to up-to-date databases. The doubling time of GenBank is currently ~16 months. It is important to search the most recent version of the database.

The BLAST (Basic Local Alignment Search Tool) family of sequence similarity search programs satisfies the above criteria. In short, users input either a nucleotide or amino acid query sequence, and search a nucleotide or amino acid sequence database. The program returns a list of the sequence "hits," alignments to the query sequence, and statistical values. This unit describes how to choose an appropriate BLAST program and database, perform the search, and interpret the results.

ACCESSING BLAST PROGRAMS AND DOCUMENTATION

The National Center for Biotechnology Information (NCBI) currently supports two versions of BLAST free of charge: BLAST 2.0 (gapped BLAST) and Position-Specific Iterated BLAST (PSI-BLAST). BLAST 2.0 is the standard version of BLAST, which allows a user to search a sequence database with a nucleotide or protein sequence of interest. BLAST 2.0 places gaps into the query and target sequences so that separate areas of similarity between the two sequences can be returned as one hit. PSI-BLAST is an iterative BLAST search, which is optimized for finding distantly related sequences.

Other BLAST programs are also available from the NCBI Web page. "BLAST 2 sequences" uses the BLAST search engine to produce an alignment of two sequences entered by the user. On the Specialized BLAST pages, researchers can use the BLAST engine to search sequences that are not in GenBank. At present, these databases include unfinished microbial genomes, *P. falciparum* (the human malaria parasite), and tentative

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human consensus (THC) sequences from The Institute for Genomic Research (TIGR). The content of these pages is, however, subject to change.

The easiest and most popular way to access the BLAST suite of programs is through the NCBI World Wide Web site, at *http://www.ncbi.nlm.nih.gov/BLAST/*. All versions of BLAST are accessible from this site, and can be used to query all sequence databases available at the NCBI. Documentation, which includes an overview of BLAST, BLAST frequently asked questions (FAQs), a "What's New" page, the BLAST manual, and a list of references, is also available here.

For users who want to run BLAST against private local databases or downloaded copies of NCBI databases, the NCBI offers a stand-alone version of the BLAST program. BLAST binaries and documentation are provided for the latest versions of IRIX, Solaris, DEC OSF1, and Win32 systems. BLAST 2.0 executables may be found on the NCBI anonymous FTP server at *ftp://ncbi.nlm.nih.gov/blast/executables/*.

BLAST can also be run as a client-server program, in which the user installs client software on a local machine that communicates across the network with a server at NCBI. This setup is useful for researchers who run large numbers of searches on NCBI databases, because they can automate the process to run on their local computer. The BLAST client may be found on the NCBI anonymous FTP server at *ftp://ncbi.nlm.nih.gov/blast/network/netblast*.

The NCBI BLAST e-mail server is the best option for people without convenient access to the Web. A similarity search can be performed by sending a properly formatted e-mail message containing the nucleotide or protein query sequence to *blast@ncbi.nlm.nih.gov*. The query sequence is compared against a specified database and the results are returned in an e-mail message. For more information on formulating e-mail BLAST searches, please send a message consisting of the word HELP to the same address, *blast@ncbi.nlm.nih.gov*.

This unit concentrates on the BLAST searches which can be performed from the NCBI Web site. The other implementations are mainly for advanced users of BLAST, or those with special needs. For any of these services, questions should be directed to *blast-help@ncbi.nlm.nih.gov*.

INTRODUCTION TO BLAST

Basic Versus Advanced BLAST Searches

BLAST 2.0 is available in a basic or advanced version. In both versions, the user can select the type of BLAST program and the database to be searched, and choose whether to filter the query sequence to mask low-complexity regions (see below). The advanced version allows the user to change parameters as well. For most researchers, the Basic version, which uses the default parameters, is adequate. For a discussion of BLAST parameters, see Appendix A at the end of this unit.

BLAST Programs

Five types of the BLAST program have been developed to support sequence similarity searching using a variety of nucleotide and protein sequence queries and databases. These programs are listed and described in Table 2.5.1. The type of BLAST search to be carried out is dependent on the type of information that is desired.

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Table 2.5.1 BLAST Search Programs

Program	Query sequence	Database sequence	Comments
BLASTP	Protein	Protein	Can be run in standard mode or in a more sensitive iterative mode (PSI-BLAST), which uses the previous search results to build a profile for subsequent rounds of similarity searching.
BLASTN	Nucleotide (both strands)	Nucleotide	Parameters optimized for speed, not sensitivity; not intended for finding distantly related coding sequences. Automatically checks complementary strand of query.
BLASTX	Nucleotide (six-frame translation)	Protein	Very useful for preliminary data containing potential frameshift errors (ESTs, HTGs, and other "single-pass" sequences).
TBLASTN	Protein	Nucleotide (six-frame translation)	Essential for searching protein queries against EST database. Often useful for finding undocumented open reading frames or frameshift errors in database sequences.
TBLASTX	Nucleotide (six-frame translation)	Nucleotide (six-frame translation)	Should be used only if BLASTN and BLASTX produce no results. Restricted for search against EST, STS, HTGS, GSS, and Alu databases.

NCBI Databases

One frequent mistake in sequence similarity searching is failure to search an up-to-date database. The NCBI produces GenBank (Benson et al., 1998; *ftp://ncbi.nlm.nih.gov/genbank/gbrel.txt*) and updates it daily. It also shares data on a daily basis with the DNA Data Bank of Japan (DDBJ; Tateno et al., 1998) and the European Molecular Biology Laboratory (EMBL; Stoesser et al., 1998). A search of the NCBI databases using the BLAST Web page, client, or e-mail server guarantees access to the most recent database. The NCBI supports a number of databases for sequence similarity searching. These databases are subject to change, and a current list and description are available on the NCBI Web site at *http://www.ncbi.nlm.nih.gov/BLAST/blast_databases.html*. This section describes some of the commonly used NCBI databases. Examples demonstrating the utility of various BLAST programs and NCBI databases are given in the next section.

Peptide sequence databases for BLASTP and BLASTX

nr. The nr (nonredundant) database is the most comprehensive. GenBank, DDBJ, and EMBL are nucleotide sequence databases. If any nucleotide sequence in any of the databases is annotated with a coding sequence (CDS), this CDS appears in nr. nr also contains protein sequences obtained from PDB (sequences associated with 3-dimensional structures in the Brookhaven Protein Data Bank), Swiss-Prot (a curated database of protein sequences; Bairoch and Apweiler, 1998), PIR (Protein Identification Resource, a comprehensive collection of protein sequences; Barker et al., 1998), and PRF (Protein Research Foundation). Although nr may contain multiple copies of similar sequences, identical sequences are merged into one entry. To be merged, two sequences must have identical lengths and every residue at every position must be the same. There are nr databases for both peptide and nucleotide sequences. The peptide database is automatically selected for BLASTP and BLASTX searches.

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month. The month database receives its sequences from the same sources as nr, but contains only those sequences released within the last 30 days. All sequences in month are also present in nr.

Alu. The Alu database contains six-frame translations of representative *Alu* repeats from all *Alu* subfamilies (Claverie and Makalowski, 1994). If a query sequence containing an *Alu* repeat is used in a BLAST search of nr or month, many of the resulting high-scoring hits will also contain *Alu* sequences. It may be useful, especially with a genomic sequence query, to perform a search of the Alu database to identify the location of any *Alu* repeats that might produce high-scoring and potentially misleading hits in queries of other databases.

Nucleotide sequence databases for BLASTN, TBLASTN, and TBLASTX

nr. The nr (nonredundant) database contains all nucleotide sequences present in GenBank, EMBL, and DDBJ. It also contains nucleotide sequences obtained from PDB (sequences associated with 3-dimensional structures in the Brookhaven Protein Data Bank). nr comprises only sequences that are normally well annotated, so it does not contain expressed sequence tag (EST), sequence-tagged site (STS), genome survey sequence (GSS), or high-throughput genomic (HTG) sequences. Although nr may contain multiple copies of similar sequences, identical sequences are merged into one entry. To be merged, two sequences must have identical lengths and every nucleotide at every position must be the same.

month. The month database contains all nucleotide sequences present in GenBank, EMBL, DDBJ, and PDB that were released within the last 30 days. Unlike nr, it also contains EST, STS, GSS, and HTG sequences released within the last month.

EST. EST accesses a nonredundant copy of all ESTs present in GenBank, EMBL, and DDBJ (Boguski et al., 1993). ESTs are short sequences, a few hundred nucleotides in length, which are derived by partial, single-pass sequencing of inserts of randomly selected cDNA clones (Adams et al., 1991). Since the number of ESTs is increasing rapidly, it is an important database to search for novel cDNAs. As of August, 1998, ~70% of the sequences in GenBank were ESTs; of these, 61% were from human, 20% from mouse.

STS. STS contains a nonredundant copy of all STSs present in GenBank, EMBL, and DDBJ. An STS is a short unique genomic sequence that is used as a sequence landmark for genomic mapping efforts (Olson et al., 1989). As of August, 1998, 83% of the sequences in the STS database were from human.

HTGS. HTGS contains "unfinished" DNA sequences generated by the high-throughput sequencing centers (Ouellette and Boguski, 1997). A typical HTG record might consist of all the first-pass sequence data generated from a single cosmid, BAC, YAC, or P1 clone. The record is composed of two or more sequence fragments that have a total length of ≥2 kb and contain one or more gaps. The sequences are normally updated by the sequencing centers as more data become available. A single accession number is assigned to this collection of sequences. The accession number does not change as the record is updated, and only the most recent version of the record remains in GenBank. Phase 1 HTG sequences are unordered, unoriented contigs with gaps. Phase 2 HTG sequences are ordered, oriented contigs with or without gaps. All HTG records contain a prominent warning that the sequence data is unfinished and may contain errors. When a record is considered finished, it becomes a Phase 3 HTG and is moved to the nr database with the same accession number. HTGS is a valuable source of new genomic sequences not yet in nr.

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GSS. GSS includes short, single-pass genomic data identified by various means (Smith et al., 1994). Many of the sequences have been mapped. As of August, 1998, 80% of the sequences in GSS were from human, 14% from *Arabidopsis thaliana.*

*Alu.*The Alu database contains representative *Alu* repeats from all *Alu* subfamilies (Claverie and Makalowski, 1994). If a query sequence containing an *Alu* repeat is used in a BLAST search of the above nucleotide databases, many of the resulting high-scoring hits will also contain *Alu* sequences. It may be useful, especially with a genomic sequence query, to perform a search of the Alu database to identify the location of any *Alu* repeats that might produce high-scoring and potentially misleading hits in queries of other databases.

Vector. The Vector database contains nucleotide sequences of a number of standard cloning vectors. New sequences should be screened against the Vector database to assure that they do not contain any vector contamination.

Mito. The Mito database contains representative mitochondrial sequences from many families. Nuclear-derived sequences may be screened against the Mito database to assure that they do not contain any mitochondrial contamination.

Formatting the Query Sequence

Users of the BLAST Web page can initiate a search either by entering the sequence itself, or, if the sequence is already in the sequence database, by entering the accession number or gi (see Appendix B at the end of this unit). The preferred format for entering new sequences is the so-called FASTA format; however, if the sequence is not in FASTA format or the sequence is interspersed with numbers and spaces, BLAST will still accept the query. A sequence in FASTA format begins with a single-line description, followed by lines of sequence data. The description line is distinguished from the sequence data by a > symbol (greater than) in the first column. An example sequence in FASTA format is:

```
>aaseq Human choroideremia protein
MADNLPTEFDVVIIGTGLPESILAAACSRSGQRVLHIDSRSYYGGNWASFSFSGLLSWLKEYQQNNDIGE
ESTVVWQDLIHETEEAITLRKKDETIQHTEAFPYASQDMEDNVEEIGALQKNPSLGVSNTFTEVLDSALP
EESQLSYFNSDEMPAKHTQKSDTEISLEVTDVEESVEKEKYCGDKTCMHTVSDKDGDKDESKSTVEDKAD
EPIRNRITYSQIVKEGRRFNIDLVSKLLYSQGLLIDLLIKSDVSRYVEFKNVTRILAFREGKVEQVPCSR
ADVFNSKELTMVEKRMLMKFLTFCLEYEQHPDEYQAFRQCSFSEYLKTKKLTPNLQHFVLHSIAMTSESS
CTTIDGLNATKNFLQCLGRFGNTPFLFPLYGQGEIPQGFCRMCAVFGGIYCLRHKVQCFVVDKESGRCKA
IIDHFGQRINAKYFIVEDSYLSEETCSNVQYKQISRAVLITDQSILKTDLDQQTSILIVPPAEPGACAVR
VTELCSSTMTCMKDTYLVHLTCSSSKTAREDLESVVKKLFTPYTETEINEEELTKPRLLWALYFNMRDSS
GISRSSYNGLPSNVYVCSGPDCGLGNEHAVKQAETLFQEIFPTEEFCPPPPNPEDIIFDGDDKQPEAPGT
NNVVMAKLESSEESKNLESPEKHLQN
```

Alternatively, the user can enter the database accession number or gi. The above sequence is in fact already in the database with a gi identifier of 116365 and a Swiss-Prot accession number of P26374. Either of these sequence identifiers can be entered on the BLAST page. The type of identifier (for nucleotide or protein sequence) must match the type of query sequence used in that search (Ostell and Kans, 1998; Ouellette, 1998). Sequences in manuscripts are often referred to by their GenBank accession number; however, this accession number refers to a nucleotide (not protein) sequence, and cannot be used to initiate a BLASTP or TBLASTN search, both of which require a protein sequence query. The identifier of the protein sequence encoded by a nucleotide accession number can be obtained by searching the NCBI's Entrez nucleotide database, at *http://www.ncbi.nlm.nih.gov/Entrez.*

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Filtering Sequences

Both nucleotide and protein sequences may contain regions of low complexity, i.e., regions with homopolymeric tracts, short-period repeats, or segments enriched in one or only a few residues. Such low-complexity regions commonly give spuriously high BLAST scores that reflect compositional bias rather than significant position-by-position alignment (Altschul et al., 1994). For example, two protein sequences that contain low-complexity regions rich in the same amino acids may produce high-scoring alignments in those regions even though other parts of the proteins are entirely dissimilar. Because these alignments do not reflect a common ancestry, no functional inference is justified despite their high statistical significance. Filtering the query sequence (that is, replacing the repeated sequence with strings of n for nucleotide sequence or X for protein sequence) can eliminate potentially confounding matches, such as hits to low-complexity, proline-rich regions or poly(A) tails present in the database.

By default, all searches performed through the NCBI BLAST Web page automatically filter the query sequence, as do the BLAST clients, e-mail server, and stand-alone programs. However, filtering can be turned off, even on the Basic BLAST page. Filtered sequence is represented in the final BLAST report as a string of n or X (e.g., nnnnnnnnnn or XXXXXXXXX). BLASTN queries are filtered with DUST (R.L. Tatusov and D.J. Lipman, pers. comm.). Other BLAST queries use SEG (Wootton and Federhen, 1993, 1996).

Viewing the BLAST Results

The NCBI BLAST Web page can return results in one of three ways. The default is to display the results in the browser window from which the user initiated the search. The document will have hypertext links that make it easier to analyze the results. BLAST queries are processed in the order in which they are received, except that less computationally intensive jobs (e.g., BLASTN, BLASTP, and smaller databases such as month) are given priority. In the middle of the afternoon, the BLAST server may be busy. Thus, it is sometimes more efficient to receive the BLAST results by e-mail. E-mail results are sent either as plain text or in HTML format. The HTML-formatted results must be opened in a Web browser, and the resulting document contains hypertext links.

EXAMPLES OF BLAST SEARCHES

In this section, interpretation of BLAST results is explained using examples for BLASTP, BLASTX, TBLASTN, BLASTN, and PSI-BLAST. An example is not shown for TBLASTX, as it is a tool of last resort and not useful for the majority of users. The first example, for BLASTP, contains general information, and should be read by all BLAST users. GenBank increases in size by thousands of sequences every week; thus, results obtained from running the same searches again will differ from those shown in the examples.

BLASTP

The BLASTP program compares a protein query to a protein database. A typical search is shown in Figure 2.5.1. The Swiss-Prot database has been selected and the program has been changed to BLASTP. A query in FASTA format has been entered in the input box. The query is the human choroideremia protein, implicated in hereditary blindness (Seabra et al., 1993).

The top of the results page for this search is shown in Figure 2.5.2. It begins with some header information about the type of program (BLASTP), the version (2.0.5), and a release

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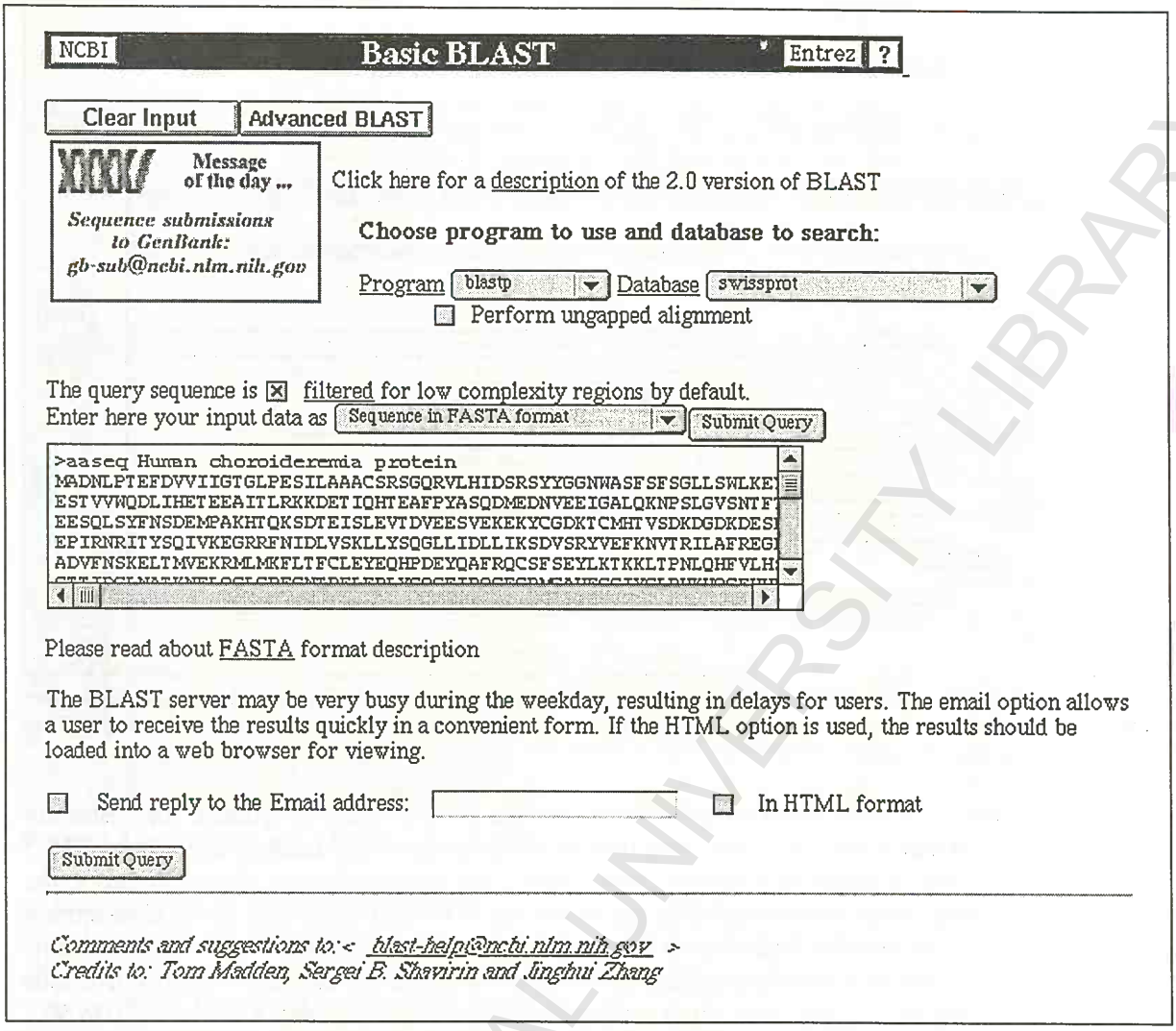

Figure 2.5.1 Submitting a BLASTP search using the NCBI's World Wide Web interface.

```
BLASTP 2.0.5 [May-5-1998]


Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Query= aaseq Human choroideremia protein
       (656 letters)

Database: Non-redundant SwissProt sequences
          74,596 sequences; 26,848,718 total letters

Searching.....................................................done

If you have any problems or questions with the results of this search
please refer to the BLAST FAQs
```

Figure 2.5.2 Example of the top portion of a BLASTP report.

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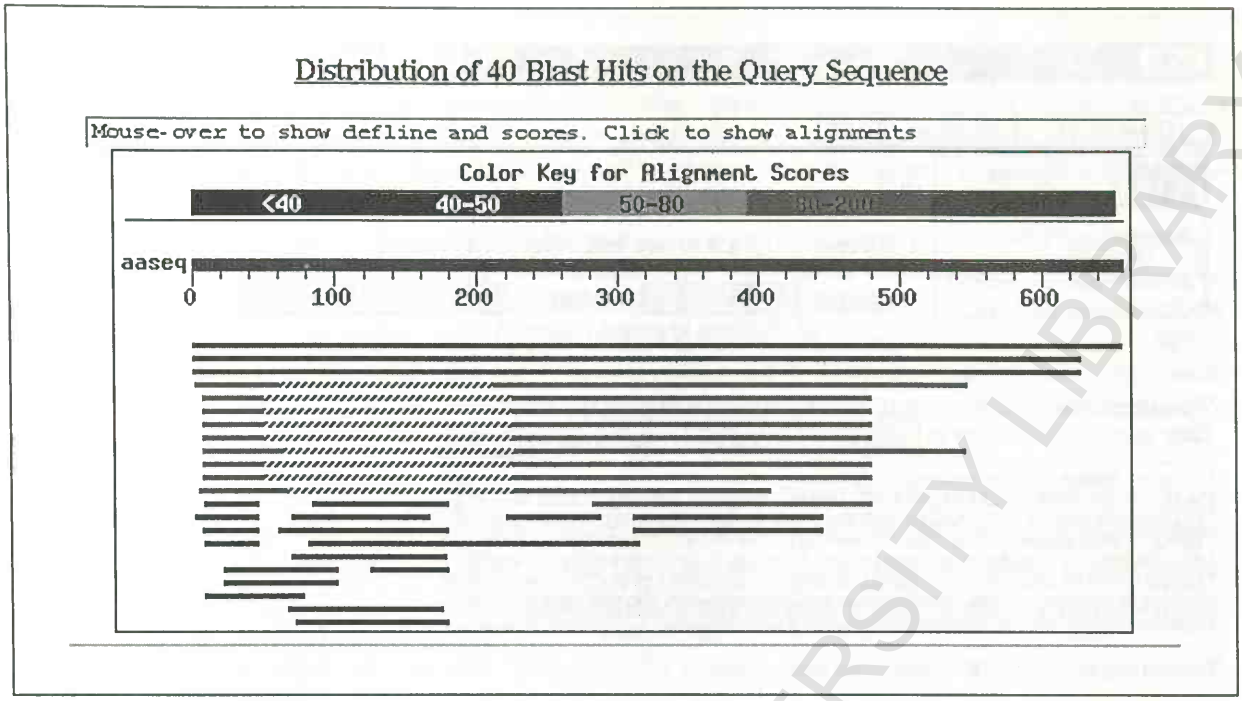

Figure 2.5.3 Example of the graphical view of a BLASTP report. The bars are color coded by the strength of the database match. The strongest matches (those with a bit score >200) are red, followed by pink (bit score 80 to 200), green (50 to 80), blue (40 to 50), and black (<40).

date. The version and release date will change as the program is updated. Also listed are a reference, the query definition line, and a summary of the database used. Figure 2.5.3 presents a graphical overview of the results. The database hits are shown aligned to the query, which is the numbered bar at the top. The next three bars show high-scoring database matches that align to the query sequence throughout its length. The next nine bars show lower-scoring matches that align to two regions of the query—one from residue 3 or 4 of the query up to about residue 60, and the other from about residues 220 to 500. For these nine bars the solid segments (on the left and right) indicate aligned regions; the cross-hatched segment indicates that the two alignments (i.e., the solid portions) are from the same database sequence, but that there is no alignment where the bar is cross-hatched. If there is no cross-hatching between two or more bars in the same row (i.e., the thirteenth row), this denotes different alignments with unrelated database sequences. These bars are presented on the same line to conserve space in the graphical view. Moving the cursor over a bar in the graphic ("mousing over") causes the identifier and definition line of the database match to be shown in the window. If the results of a BLAST search are sent by e-mail, this graphical view is not included.

The hit list produced by the BLASTP search is shown in Figure 2.5.4. Each line of the hit list is composed of four fields. The first field contains the database designation, accession number, and locus name for the matched sequence, separated by vertical bars (see Appendix B at the end of this unit for more information). The second field contains a brief textual description of the sequence, the definition line. The content of the definition line varies between and within databases, but usually includes information on the organism from which the sequence was derived, the type of sequence (e.g., mRNA or DNA), and some information about function or phenotype. The definition line is often truncated in the hit list to keep the display compact, but is fully displayed in association with the local alignments (see below). The third field contains the alignment score in bits. Higher scoring hits are found at the top of the list. In very general terms, this score is calculated from a formula that takes into account the alignment of similar or identical

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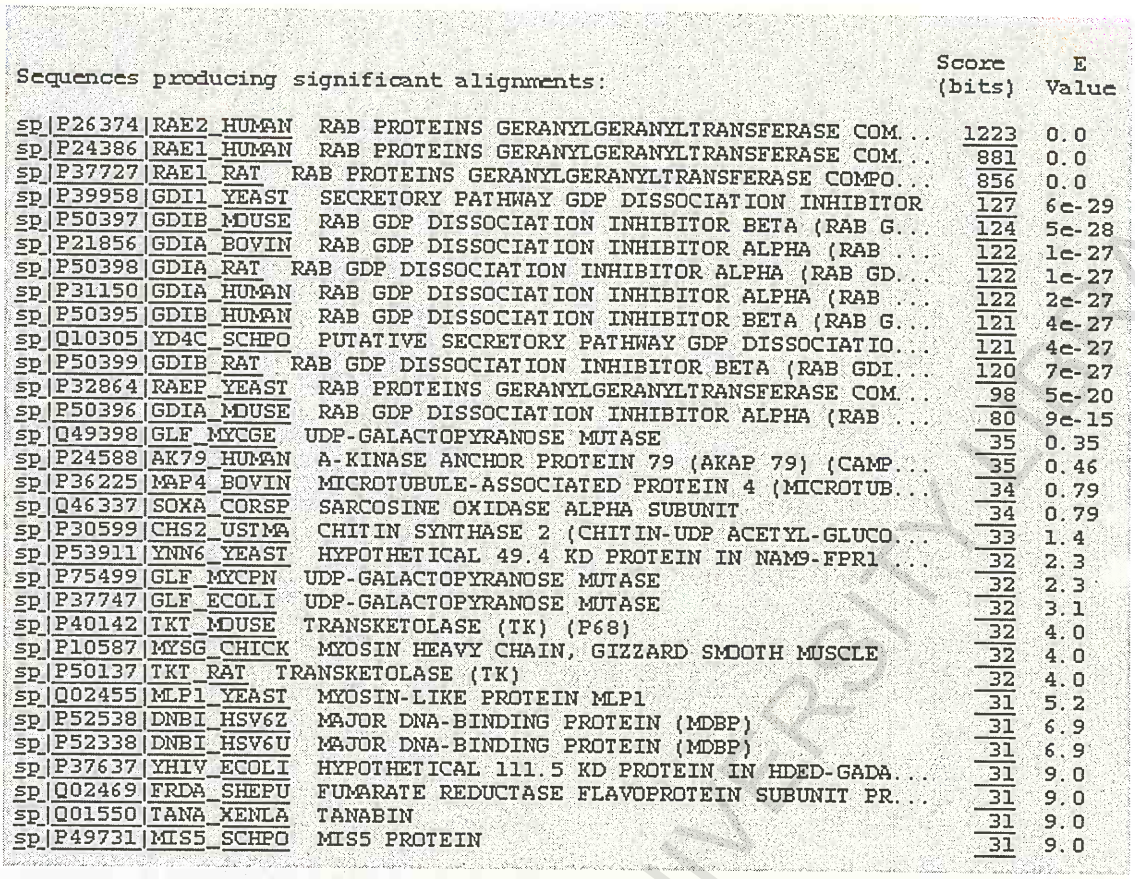

```
Sequences producing significant alignments:                          Score     E
                                                                     (bits)  Value

sp|P26374|RAE2_HUMAN  RAB PROTEINS GERANYLGERANYLTRANSFERASE COM...   1223  0.0
sp|P24386|RAE1_HUMAN  RAB PROTEINS GERANYLGERANYLTRANSFERASE COM...    881  0.0
sp|P37727|RAE1_RAT  RAB PROTEINS GERANYLGERANYLTRANSFERASE COMPO...    856  0.0
sp|P39958|GDI1_YEAST  SECRETORY PATHWAY GDP DISSOCIATION INHIBITOR     127  6e-29
sp|P50397|GDIB_MOUSE  RAB GDP DISSOCIATION INHIBITOR BETA (RAB G...    124  5e-28
sp|P21856|GDIA_BOVIN  RAB GDP DISSOCIATION INHIBITOR ALPHA (RAB ...    122  1e-27
sp|P50398|GDIA_RAT  RAB GDP DISSOCIATION INHIBITOR ALPHA (RAB GD...    122  1e-27
sp|P31150|GDIA_HUMAN  RAB GDP DISSOCIATION INHIBITOR ALPHA (RAB ...    122  2e-27
sp|P50395|GDIB_HUMAN  RAB GDP DISSOCIATION INHIBITOR BETA (RAB G...    121  4e-27
sp|Q10305|YD4C_SCHPO  PUTATIVE SECRETORY PATHWAY GDP DISSOCIATIO...    121  4e-27
sp|P50399|GDIB_RAT  RAB GDP DISSOCIATION INHIBITOR BETA (RAB GDI...    120  7e-27
sp|P32864|RAEP_YEAST  RAB PROTEINS GERANYLGERANYLTRANSFERASE COM...     98  5e-20
sp|P50396|GDIA_MOUSE  RAB GDP DISSOCIATION INHIBITOR ALPHA (RAB ...     80  9e-15
sp|Q49398|GLF_MYCGE  UDP-GALACTOPYRANOSE MUTASE                        35  0.35
sp|P24588|AK79_HUMAN  A-KINASE ANCHOR PROTEIN 79 (AKAP 79) (CAMP...    35  0.46
sp|P36225|MAP4_BOVIN  MICROTUBULE-ASSOCIATED PROTEIN 4 (MICROTUB...    34  0.79
sp|Q46337|SOXA_CORSP  SARCOSINE OXIDASE ALPHA SUBUNIT                  34  0.79
sp|P30599|CHS2_USTMA  CHITIN SYNTHASE 2 (CHITIN-UDP ACETYL-GLUCO...    33  1.4
sp|P53911|YNN6_YEAST  HYPOTHETICAL 49.4 KD PROTEIN IN NAM9-FPR1 ...    32  2.3
sp|P75499|GLF_MYCPN  UDP-GALACTOPYRANOSE MUTASE                        32  2.3
sp|P37747|GLF_ECOLI  UDP-GALACTOPYRANOSE MUTASE                        32  3.1
sp|P40142|TKT_MOUSE  TRANSKETOLASE (TK) (P68)                          32  4.0
sp|P10587|MYSG_CHICK  MYOSIN HEAVY CHAIN, GIZZARD SMOOTH MUSCLE        32  4.0
sp|P50137|TKT_RAT  TRANSKETOLASE (TK)                                  32  4.0
sp|Q02455|MLP1_YEAST  MYOSIN-LIKE PROTEIN MLP1                         31  5.2
sp|P52538|DNBI_HSV6Z  MAJOR DNA-BINDING PROTEIN (MDBP)                 31  6.9
sp|P52338|DNBI_HSV6U  MAJOR DNA-BINDING PROTEIN (MDBP)                 31  6.9
sp|P37637|YHIV_ECOLI  HYPOTHETICAL 111.5 KD PROTEIN IN HDED-GADA...    31  9.0
sp|Q02469|FRDA_SHEPU  FUMARATE REDUCTASE FLAVOPROTEIN SUBUNIT PR...    31  9.0
sp|Q01550|TANA_XENLA  TANABIN                                          31  9.0
sp|P49731|MIS5_SCHPO  MIS5 PROTEIN                                     31  9.0
```

Figure 2.5.4 Example of the hit list from a BLASTP report.

residues, as well as any gaps that must be introduced in order to align the sequences. A key element in this calculation is the "substitution matrix," which assigns a score for aligning any possible pair of residues. The BLOSUM-62 matrix is the default for BLAST, and it works well for most searches. The fourth field contains the Expect (*E*) value, which provides an estimate of statistical significance. *E* values reflect how many times one expects to see such a score occur by chance. A statistician would consider an *E* value <0.05 to be significant. However, an inspection of the alignments (see below) is required to determine biological significance. The maximum number of hits displayed in the hit list is set at a default value of 500. This number can be changed on the advanced BLAST page with the *descriptions* option.

For the first hit in the list, the database designation is sp (for Swiss-Prot), the accession number is P26374, the locus name is RAE2_HUMAN, the definition line begins RAB PROTEINS, the score is 1223, and the *E* value is 0.0.

The higher the score and the lower the *E* value, the more statistically significant is the hit. Note that the first thirteen hits all have very low *E* values ($<10^{-14}$) and are either RAB proteins or GDP dissociation inhibitors. The next eighteen database matches have much higher *E* values (≥0.35), meaning that roughly one match with that score would be expected by chance. Furthermore, the definition lines no longer show the same consistency. Given the combination of inconsistent definition lines and high *E* values, caution should be used in assuming an evolutionary relationship between the query and the eighteen lower-scoring database hits. It would be necessary to examine the alignments in order to make a final determination.

The pairwise alignments between the query and the database hits are displayed below the hit list. Figure 2.5.5 shows one sequence alignment between the query sequence and a

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```
sp|P37727|RAE1_RAT RAB PROTEINS GERANYLGERANYLTRANSFERASE COMPONENT A 1 (RAB ESCORT
          PROTEIN 1) (REP-1)
          Length = 650

 Score =  856 bits (2188), Expect = 0.0
 Identities = 433/632 (68%), Positives = 491/632 (77%), Gaps = 13/632 (2%)

Query: 1   MADNLPTEFDVVIIGTGLPESILAAACSRSGQRVLHIDSRSYYGGNWASFSFSGLLSWLK 60
           MADNLP++FDV++IGTGLPESI+AAACSRSGQRVLH+DSRSYYGGNWASFSFSGLLSWLK
Sbjct: 1   MADNLPSDFDVIVIGTGLPESIIAAACSRSGQRVLHVDSRSYYGGNWASFSFSGLLSWLK 60

Query: 61  EYQQNNDIGEESTVVWQDLIHETEEAITLRKKDETIQHTEAFPYASQDMEDNVEEIGALQ 120
           EYQ+NND+  E+++ WQ+ I E EEAI L  KD+TIQH E F YASQD+  +VEE GALQ
Sbjct: 61  EYQENNDVVTENSM-WQEQILENEEAIPLSSKDKTIQHVEVFCYASQDLHKDVEEAGALQ 119

Query: 121 KNPSLGVSNTFTEVLDSA----LPEESQLSYFNSDEMPAKHTQKSDTEISLEVTDVEESV 176
           KN +   S    E  ++A    LP   +     S E+PA+ +Q    E S EV D E +
Sbjct: 120 KNHASVTSAQSAEAAEAAETSCLPTAVEPLSMGSCEIPAEQSQCPGPESSPEVNDAEATG 179

Query: 177 EKEKYCGDKTCMHTVXXXXXXXXXXXXXTVEDKADEPIRNRITYSQIVKEGRRFNIDLVSK 236
           +KE                          V+D  + P +NRITYSQI+KEGRRFNIDLVS+
Sbjct: 180 KKEN--------SDAKSSTEEPSENVPKVQDNTETPKKNRITYSQIIKEGRRFNIDLVSQ 231

Query: 237 LLYSQGLLIDLLIKSDVSRYVEFKNVTRILAFREGKVEQVPCSRADVFNSKELTMVEKRM 296
           LLYS+GLLIDLLIKS+VSRY EFKN+TRILAFREG VEQVPCSRADVFNSK+LTMVEKRM
Sbjct: 232 LLYSRGLLIDLLIKSNVSRYAEFKNITRILAFREGTVEQVPCSRADVFNSKQLTMVEKRM 291

Query: 297 LMKFLTFCLEYEQHPDEYQAFRQCSFSEYLKTKKLTPNLQHFVLHSIAMTSESSCTTIDG 356
           LMKFLTFC+EYE+HPDEY+A+    +FSEYLKT+KLTPNLQ+FVLHSIAMTSE++  T+DG
Sbjct: 292 LMKFLTFCVEYEEHPDEYRAYEGTTFSEYLKTQKLTPNLQYFVLHSIAMTSETTSCTVDG 351

Query: 357 LNATKNFLQCLGRFGNTPFLFPLYGQGEIPQGFCRMCAVFGGIYCLRHKVQCFVVDKESG 416
           L ATK FLQCLGR+GNTPFLFPLYGQGE+PQ FCRMCAVFGGIYCLRH VQC VVDKES
Sbjct: 352 LKATKKFLQCLGRYGNTPFLFPLYGQGELPQCFCRMCAVFGGIYCLRHSVQCLVVDKESR 411

Query: 417 RCKAIIDHFGQRINAKYFIVEDSYLSEETCSNVQYKQISRAVLITDQSILKTDLDQQTSI 476
           +CKA+ID FGQRI +K+FI+EDSYLSE TCS VQY+QISRAVLITD S+LKTD DQQ SI
Sbjct: 412 KCKAVIDQFGQRIISKHFIIEDSYLSENTCSRVQYRQISRAVLITDGSVLKTDADQQVSI 471

Query: 477 LIVPPAEPGACAVRVTELCSSTMTCMKDTYLVHLTCSSSKTAREDLESVVKKLFTPYTET 536
           L VP  EPG+  VRV ELCSSTMTCMK TYLVHLTC SSKTAREDLE VV+KLFTPYTE
Sbjct: 472 LAVPAEEPGSFGVRVIELCSSTMTCMKGTYLVHLTCMSSKTAREDLERVVQKLFTPYTEI 531

Query: 537 EINEEELTKPRLLWALYFNMRDSSGISRSSYNGLPSNVYVCSGPDCGLGNEHAVKQAETL 596
           E   E++ KPRLLWALYFNMRDSS ISR  YN LPSNVYVCSGPD GLGN++AVKQAETL
Sbjct: 532 EAENEQVEKPRLLWALYFNMRDSSDISRDCYNDLPSNVYVCSGPDSGLGNDNAVKQAETL 591

Query: 597 FQXXXXXXXXXXXXXXXXXXXXDGDDKQPEAP 628
           FQ                    DGD  Q E P
Sbjct: 592 FQQICPNEDFCPAPPNPEDIVLDGDSSQQEVP 623
```

Figure 2.5.5 Example of a BLASTP alignment.

database hit; other alignments are omitted for brevity. The alignment is preceded by the sequence identifier, the full definition line, and the length, in amino acids, of the database sequence. Next comes the score of the match in bits (the raw score is in parentheses), the Expect value (*E* value) of the hit, the number of identical residues in the alignment (identities), the number of conservative substitutions (positives) according to the scoring system (e.g., BLOSUM-62), and, if applicable, the number of gaps in the alignment. Finally the actual alignment is shown, with the query on top and the database match labeled as subject (Sbjct). Since both the query and the match are amino acid sequences, the numbers at left and right refer to the position in the amino acid sequence. The center line between the two sequences indicates the similarities between the sequences. If the residue is identical between the query and the subject, the residue itself is shown; conservative substitutions, as judged by the substitution matrix, are indicated with a + sign. One or more dashes (–) within a sequence indicates insertions or deletions. Query residues masked for low complexity are replaced by Xs (see fourth and eleventh blocks, Fig. 2.5.5). By default, a maximum of 500 pairwise alignments are displayed. This number can be changed on the advanced BLAST page with the *alignments* option.

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Results generated on the NCBI BLAST Web page contain text hyperlinks that assist the user in navigating around the page. Clicking on a colored bar in the graphical view (Fig. 2.5.3) moves the user to the sequence alignment (Fig. 2.5.5). The database sequence identifiers, visible both in the one-line descriptions of the database matches (Fig. 2.5.4) and in the sequence alignment (Fig. 2.5.5), link to the Entrez record that describes the sequence. Entrez may provide more information about the sequence, including links to relevant publication abstracts in PubMed. The score (to the right of the one-line description; Fig. 2.5.4) provides a link within the page to the alignment between that hit and the query.

BLASTX

BLASTX searches involve a six-frame translation of a nucleotide sequence queried against a protein database (Gish and States, 1993). Researchers most often use a BLASTX search if they have sequenced a DNA molecule and do not know the reading frame of the coding sequence, the beginning or end of the open reading frame, or the function of the encoded protein. It is also useful for analyzing preliminary sequence data containing potential sequencing or frameshift errors, such as ESTs, HTGs, or GSSs. A BLASTX search of the nr protein database can quickly highlight any similarities between all the open reading frames in a nucleotide sequence and any characterized proteins.

In the example illustrated here, the mRNA sequence encoding human ataxia telangiectasia (ATM; accession number U33841; Lavin and Shiloh, 1997) was queried against the nr protein database. The output is similar to that from BLASTP. At the top (not shown) is information about the search, including the query, database, and type of BLAST program used. Next is the graphical overview representing the alignment of the database hits to the query sequence (Fig. 2.5.6), followed by a list of the hits, including their definitions, scores, and *E* values (Fig. 2.5.7 shows the top portion of the list). Pairwise alignments between the query and hit sequences follow (selected alignments are shown in Fig. 2.5.8).

The top-scoring seven hits, which have scores >2600, are to other orthologs of the ATM gene (Fig. 2.5.7). Most of these hits align with the query sequence over its entire length (in Fig. 2.5.6, the first five bars span the entire length of the query sequence; note also the longer bars in the sixth and seventh lines). The 3′ end of the ATM mRNA (carboxy terminal end of the resulting protein translation), aligns with the catalytic domain of a number of phosphatidylinositol 3-kinases or similar proteins—such as the yeast TEL1 gene (a phosphatidylinositol kinase involved in controlling telomere length), the yeast ESR1 gene (a putative phosphatidylinositol kinase required for mitotic cell growth, DNA repair, and meiotic recombination), and the yeast TOR1 protein (a phosphatidylinositol kinase required for cell cycle progression). The alignment with these proteins starts in different places depending on the protein; one group starts around nucleotide 4700 of ATM (lines 9, 10, and 15 in Fig. 2.5.6), and other groups start around nucleotide 5800 (lines 17 to 19), 6800 (lines 7, 8, and 12 to 14), and 8000 (lines 20 to 60). Such similarities have provided insight into the role of ATM in response to DNA damage and cell cycle control (Lavin and Shiloh, 1997). The ATM gene also shares similarity with viral capsid proteins around nucleotides 2500 and 6000 (short bars on lines 6 to 14). However, these hits have low scores and high *E* values (not shown) and are not likely to be significant.

The hits to the orthologs of ATM reveal the location of the coding sequence within the query nucleotide sequence. In a BLASTX output, position in the query sequence (the top line of a pairwise alignment) is described as a nucleotide position, while the position in the database hit (the bottom line of the alignment) is described as an amino acid position (Fig. 2.5.8). For example, amino acids 1 to 3066 of the mouse ortholog of ATM, gi 1469394, align with the translation of nucleotide positions 190 through 9357 of the query

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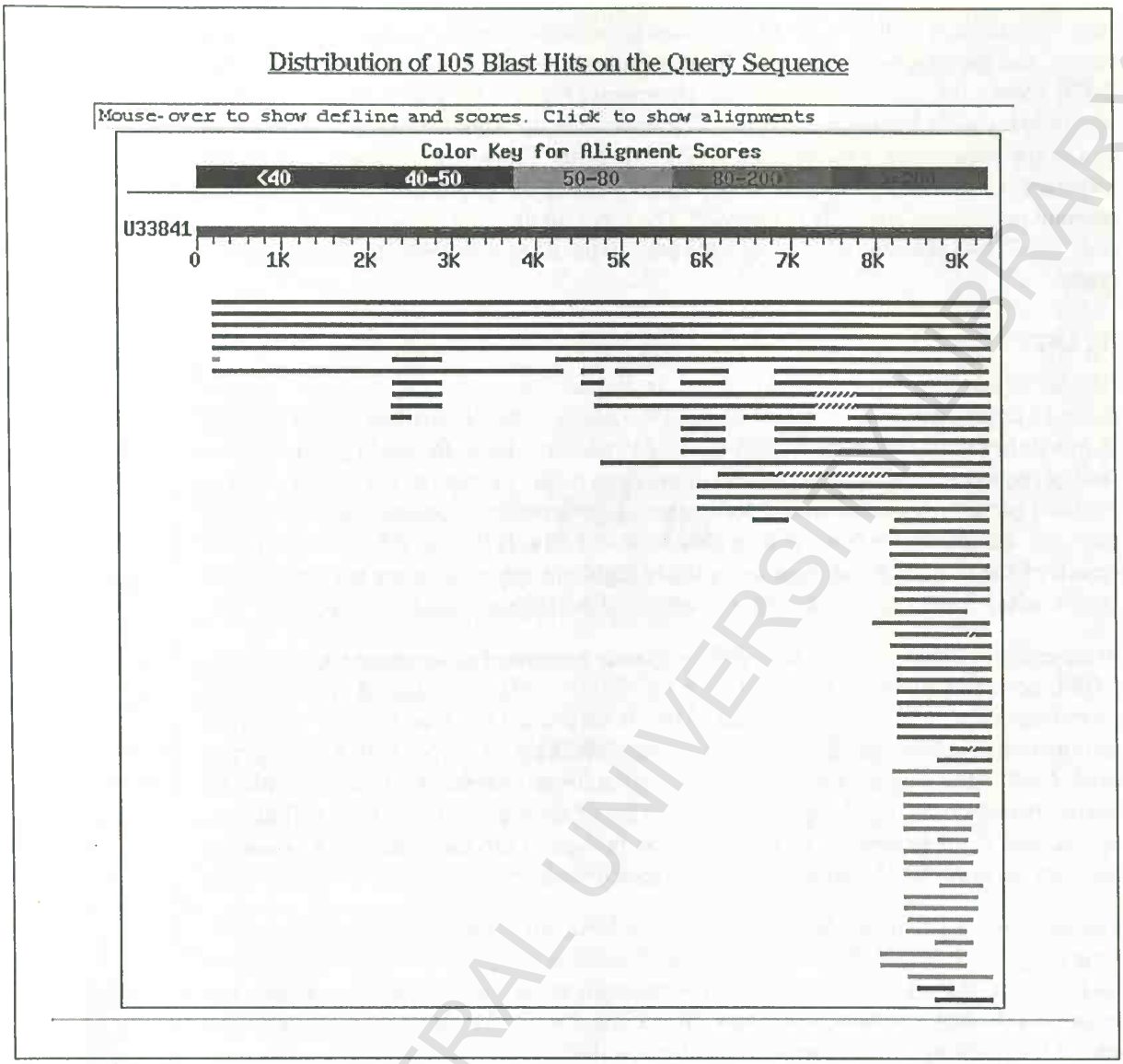

Figure 2.5.6 Example of the graphical view of a BLASTX report.

sequence. The exact location of the ATM coding sequence cannot be easily determined from the hits to the yeast sequences, as the similarity does not extend throughout the length of the protein (Fig. 2.5.8, bottom). However, the hits to the yeast sequences do provide confirmation about the phase of the reading frame, and possibly the location of specific domains conserved between humans and yeast.

TBLASTN

TBLASTN searches involve a protein sequence queried against the six-frame translation of a nucleotide sequence database. TBLASTN searches are especially useful for finding novel similarities to a protein sequence in the open reading frames from uncharacterized, sometimes lower-quality nucleotide sequences, such as those found in EST, STS, HTGS, and GSS databases. The translations of these sequences are not present in nr because the sequences are not annotated. The most common use of TBLASTN is to search for ESTs that are similar to a protein of interest. These ESTs might represent orthologs of the protein from a different species, or just additional members of a gene family.

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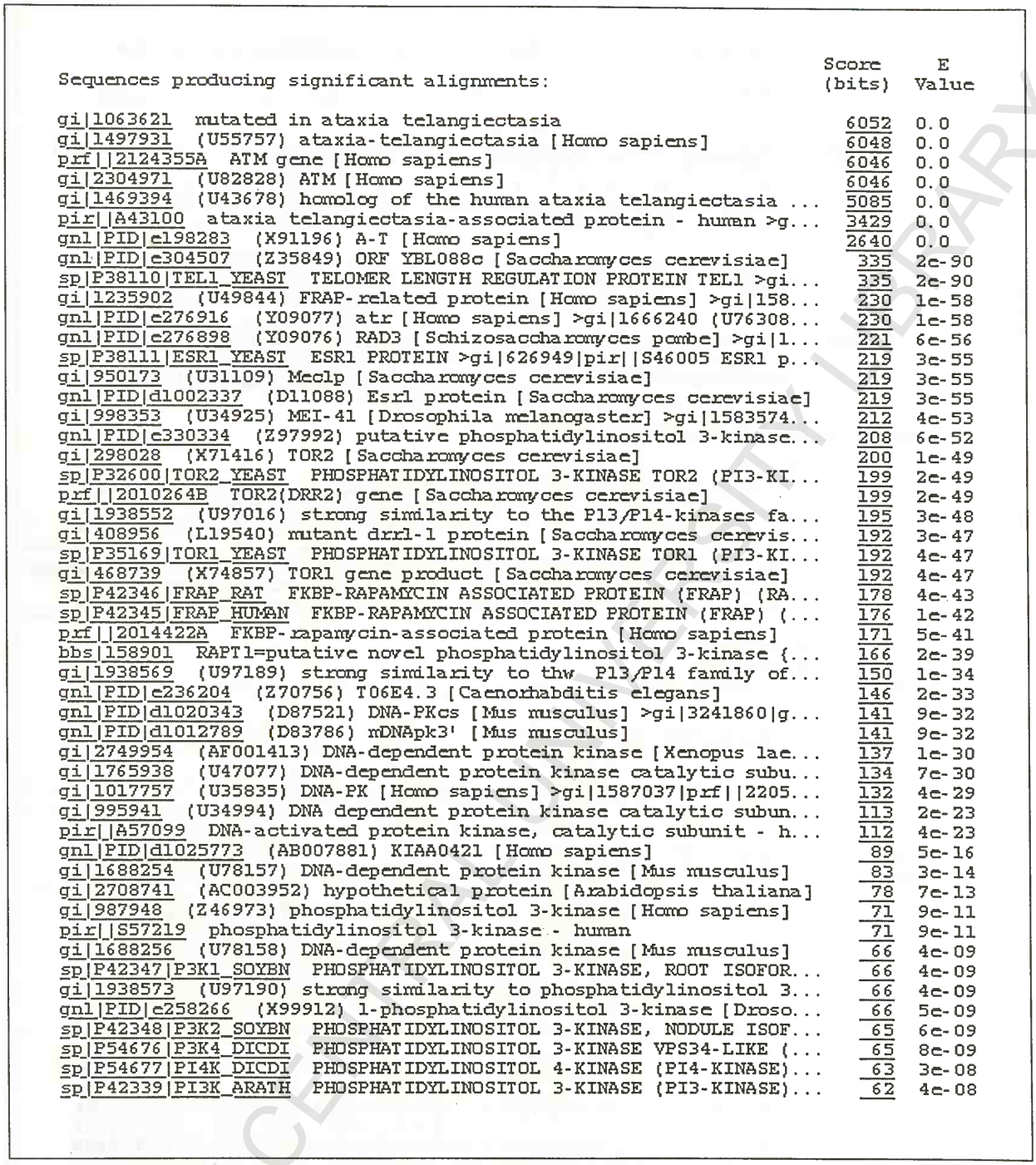

Sequences producing significant alignments:	Score (bits)	E Value
gi|1063621 mutated in ataxia telangiectasia	6052	0.0
gi|1497931 (U55757) ataxia-telangiectasia [Homo sapiens]	6048	0.0
prf||2124355A ATM gene [Homo sapiens]	6046	0.0
gi|2304971 (U82828) ATM [Homo sapiens]	6046	0.0
gi|1469394 (U43678) homolog of the human ataxia telangiectasia ...	5085	0.0
pir||A43100 ataxia telangiectasia-associated protein - human >g...	3429	0.0
gnl|PID|e198283 (X91196) A-T [Homo sapiens]	2640	0.0
gnl|PID|e304507 (Z35849) ORF YBL088c [Saccharomyces cerevisiae]	335	2e-90
sp|P38110|TEL1_YEAST TELOMER LENGTH REGULATION PROTEIN TEL1 >gi...	335	2e-90
gi|1235902 (U49844) FRAP-related protein [Homo sapiens] >gi|158...	230	1e-58
gnl|PID|e276916 (Y09077) atr [Homo sapiens] >gi|1666240 (U76308...	230	1e-58
gnl|PID|e276898 (Y09076) RAD3 [Schizosaccharomyces pombe] >gi|1...	221	6e-56
sp|P38111|ESR1_YEAST ESR1 PROTEIN >gi|626949|pir||S46005 ESR1 p...	219	3e-55
gi|950173 (U31109) Mec1p [Saccharomyces cerevisiae]	219	3e-55
gnl|PID|d1002337 (D11088) Esr1 protein [Saccharomyces cerevisiae]	219	3e-55
gi|998353 (U34925) MEI-41 [Drosophila melanogaster] >gi|1583574...	212	4e-53
gnl|PID|e330334 (Z97992) putative phosphatidylinositol 3-kinase...	208	6e-52
gi|298028 (X71416) TOR2 [Saccharomyces cerevisiae]	200	1e-49
sp|P32600|TOR2_YEAST PHOSPHATIDYLINOSITOL 3-KINASE TOR2 (PI3-KI...	199	2e-49
prf||2010264B TOR2(DRR2) gene [Saccharomyces cerevisiae]	199	2e-49
gi|1938552 (U97016) strong similarity to the P13/P14-kinases fa...	195	3e-48
gi|408956 (L19540) mutant drr1-1 protein [Saccharomyces cerevis...	192	3e-47
sp|P35169|TOR1_YEAST PHOSPHATIDYLINOSITOL 3-KINASE TOR1 (PI3-KI...	192	4e-47
gi|468739 (X74857) TOR1 gene product [Saccharomyces cerevisiae]	192	4e-47
sp|P42346|FRAP_RAT FKBP-RAPAMYCIN ASSOCIATED PROTEIN (FRAP) (RA...	178	4e-43
sp|P42345|FRAP_HUMAN FKBP-RAPAMYCIN ASSOCIATED PROTEIN (FRAP) (...	176	1e-42
prf||2014422A FKBP-rapamycin-associated protein [Homo sapiens]	171	5e-41
bbs|158901 RAPT1=putative novel phosphatidylinositol 3-kinase (...	166	2e-39
gi|1938569 (U97189) strong similarity to thw P13/P14 family of...	150	1e-34
gnl|PID|e236204 (Z70756) T06E4.3 [Caenorhabditis elegans]	146	2e-33
gnl|PID|d1020343 (D87521) DNA-PKcs [Mus musculus] >gi|3241860|g...	141	9e-32
gnl|PID|d1012789 (D83786) mDNApk3' [Mus musculus]	141	9e-32
gi|2749954 (AF001413) DNA-dependent protein kinase [Xenopus lae...	137	1e-30
gi|1765938 (U47077) DNA-dependent protein kinase catalytic subu...	134	7e-30
gi|1017757 (U35835) DNA-PK [Homo sapiens] >gi|1587037|prf||2205...	132	4e-29
gi|995941 (U34994) DNA dependent protein kinase catalytic subun...	113	2e-23
pir||A57099 DNA-activated protein kinase, catalytic subunit - h...	112	4e-23
gnl|PID|d1025773 (AB007881) KIAA0421 [Homo sapiens]	89	5e-16
gi|1688254 (U78157) DNA-dependent protein kinase [Mus musculus]	83	3e-14
gi|2708741 (AC003952) hypothetical protein [Arabidopsis thaliana]	78	7e-13
gi|987948 (Z46973) phosphatidylinositol 3-kinase [Homo sapiens]	71	9e-11
pir||S57219 phosphatidylinositol 3-kinase - human	71	9e-11
gi|1688256 (U78158) DNA-dependent protein kinase [Mus musculus]	66	4e-09
sp|P42347|P3K1_SOYBN PHOSPHATIDYLINOSITOL 3-KINASE, ROOT ISOFOR...	66	4e-09
gi|1938573 (U97190) strong similarity to phosphatidylinositol 3...	66	4e-09
gnl|PID|e258266 (X99912) 1-phosphatidylinositol 3-kinase [Droso...	66	5e-09
sp|P42348|P3K2_SOYBN PHOSPHATIDYLINOSITOL 3-KINASE, NODULE ISOF...	65	6e-09
sp|P54676|P3K4_DICDI PHOSPHATIDYLINOSITOL 3-KINASE VPS34-LIKE (...	65	8e-09
sp|P54677|PI4K_DICDI PHOSPHATIDYLINOSITOL 4-KINASE (PI4-KINASE)...	63	3e-08
sp|P42339|PI3K_ARATH PHOSPHATIDYLINOSITOL 3-KINASE (PI3-KINASE)...	62	4e-08

Figure 2.5.7 Example of the hit list from a BLASTX report.

The following example shows a TBLASTN search of HTGS, a database that contains unfinished and uncharacterized genomic sequences generated by genome sequencing centers. Since HTGs are genomic and often contain introns, the results can be more difficult to interpret than those from an EST search. However, HTGs can also provide insight into the chromosomal organization of genes.

In this case, the six-frame translation of HTGS is searched with the amino acid sequence of the disintegrin-like domain of mouse ADAM 1 (Wolfsberg et al., 1995). TBLASTN output is similar to that of BLASTP and BLASTX. Figure 2.5.9 shows the graphical overview of the alignment, Figure 2.5.10 shows the list of database hits, and Figure 2.5.11

```
gi|1469394 (U43678) homolog of the human ataxia telangiectasia gene [Mus
           musculus]
           Length = 3066

 Score = 5085 bits (13044), Expect = 0.0
 Identities = 2526/3056 (82%), Positives = 2751/3056 (89%), Gaps = 14/3056 (0%)

Query: 190  MSLVLNDLLICCRQLEHDRATERKKEVEKFKRLIRDPETIKHLDRHSDSKQGKYLNWDAV 369
            MSL LNDLLICCRQLEHDRATER+KEV+KFKRLI+DPET++HLDRHSDSKQGKYLNWDAV
Sbjct: 1    MSLALNDLLICCRQLEHDRATERRKEVDKFKRLIQDPETVQHLDRHSDSKQGKYLNWDAV 60

Query: 370  FRFLQKYIQKETECLRIAKPNVSASTQASRQKKMQEISSLVKYFIKCANRRAPRLKCQEL 549
            FRFLQKYIQKE E LR AK NVSA+TQ+SRQKKMQEISSLV+YFIKCAN+RAPRLKCQ+L
Sbjct: 61   FRFLQKYIQKEMESLRTAKSNVSATTQSSRQKKMQEISSLVRYFIKCANKRAPRLKCQDL 120

Query: 550  LNYIMDTVKDSSNGAIYGADCSNILLKDILSVRKYWCEISQQQWLELFSVYFRLYLKPSQ 729
            LNY+MDTVKDSSNG  YGADCSNILLKDILSVRKYWCE+SQQQWLELFS+YFRLYLKPSQ
Sbjct: 121  LNYVMDTVKDSSNGLTYGADCSNILLKDILSVRKYWCEVSQQQWLELFSLYFRLYLKPSQ 180
```

[middle portion of alignment not shown]

```
Query: 8968 FRRCCEKTMEVMRNSQETLLTIVEVLLYDPLFDWTMNPLKALYLQQRPEDETELHPTLNA 9147
            FRRCCEKTMEVMR+SQETLLTIVEVLLYDPLFDWTMNPLKALYLQQRPEDE++LH T NA
Sbjct: 2937 FRRCCEKTMEVMRSSQETLLTIVEVLLYDPLFDWTMNPLKALYLQQRPEDESDLHSTPNA 2996

Query: 9148 DDQECKRNLSDIDQSFDKVAERVLMRLQEKLKGVEEGTVLSVGGQVNLLIQQAIDPKNLS 9327
            DDQECK++LSD DQSF+KVAERVLMRLQEKLKGVEEGTVLSVGGQVNLLIQQA+DPKNLS
Sbjct: 2997 DDQECKQSLSDTDQSFNKVAERVLMRLQEKLKGVEEGTVLSVGGQVNLLIQQAMDPKNLS 3056

Query: 9328 RLFPGWKAWV 9357
            RLFPGWKAWV
Sbjct: 3057 RLFPGWKAWV 3066
```

[intervening alignments not shown]

```
gi|468739 (X74857) TOR1 gene product [Saccharomyces cerevisiae]
          Length = 2470

 Score =  192 bits (482), Expect = 4e-47
 Identities = 124/397 (31%), Positives = 214/397 (53%), Gaps = 19/397 (4%)

Query: 8155 LKNLEDVVVPTMEIKVDHTGEY---GNLVTIQSFKAEFRLAGGVNLPKIIDCVGSDGKER 8325
           L+++   ++ T ++++  G Y      + I  F+  F +      P+     GSDGK+
Sbjct: 2060 LQHVSPQLLATHDLELAVPGTYFPGKPTIRIAKFEPLFSVISSKQRPRKFSIKGSDGKDY 2119

Query: 8326 RQLVKGRDDLRQDAVMQQVFQMCNTLLQRNTETRKRKLTICTYKVVPLSQRSGVLEWCTG 8505
           + ++KG +D+RQD+++ Q+F + NTLL+ ++E  KR L I  Y  +PLS +SG+L W
Sbjct: 2120 KYVLKGHEDIRQDSLVMQLFGLVNTLLKNDSECFKRHLDIQQYPAIPLSPKSGLLGWVPN 2179

Query: 8506 TVPIGEEFLVNNEDGAHKRYRPNDFSAFQCQKKMMEVQKKSFEEKYEVFMDVCQNF--QPV 8679
           +    + + D   + P +   +   + + +  +K EVF     N   Q +
Sbjct: 2180 SDTFHVLIREHRDA---KKIPLNIEHWVMLQMAPDYENLTLLQKIEVFTYALDNTKGQDL 2236

Query: 8680 FRYFCMEKFLDPAIWFEKRLAYTRSVATSSIVGYILGLGDRHVQNILINEQSAELVHIDL 8859
           ++   + K      W E+R  YTRS+A  S+ GYILGLGDRH  N++++  + +++HID
Sbjct: 2237 YKILWL-KSRSSETWLERRTTYTRSLAVMSMTGYILGLGDRHPSNLMLDRITGKVIHIDF 2295

Query: 8860 GVAFEQGKILPT-PETVPFRLTRDIVDGMGITGVEGVFRRCCEKTMEVMRNSQETLLTIV 9036
           G  FE   +    PE VPFRLTR +   M ++G+EG FR  CE  M V+R+++E+L+ I+
Sbjct: 2296 GDCFEAAILREKYPEKVPFRLTRMLTYAMEVSGIEGSFRITCENVMRVLRDNKESLMAIL 2355

Query: 9037 EVLLYDPLFDWTMNPLKALYLQQRPEDETELHPTLNADDQECKRNLSDIDQSFDKVAER- 9213
           E    DPL  W  +        Q+  ++T +   L    +  ++    ++++ +  AE+
Sbjct: 2356 EAFALDPLIHWGFD-----LPPQKLTEQTGIPLPLINPSELLRKGAITVEEAANMEAEQQ 2410

Query: 9214 ----------VLMRLQEKLKG--VEEGTVLSVGGQVNLLIQQAIDPKNLSRLFPGW 9345
                      VL R+ +KL G  ++    L V  QV+ LIQQA    + L + + GW
Sbjct: 2411 NETRNARAMLVLRRITDKLTGNDIKRFNELDVPEQVDKLIQQATSIERLCQHYIGW 2466
```

Figure 2.5.8 Example of selected BLASTX alignments.

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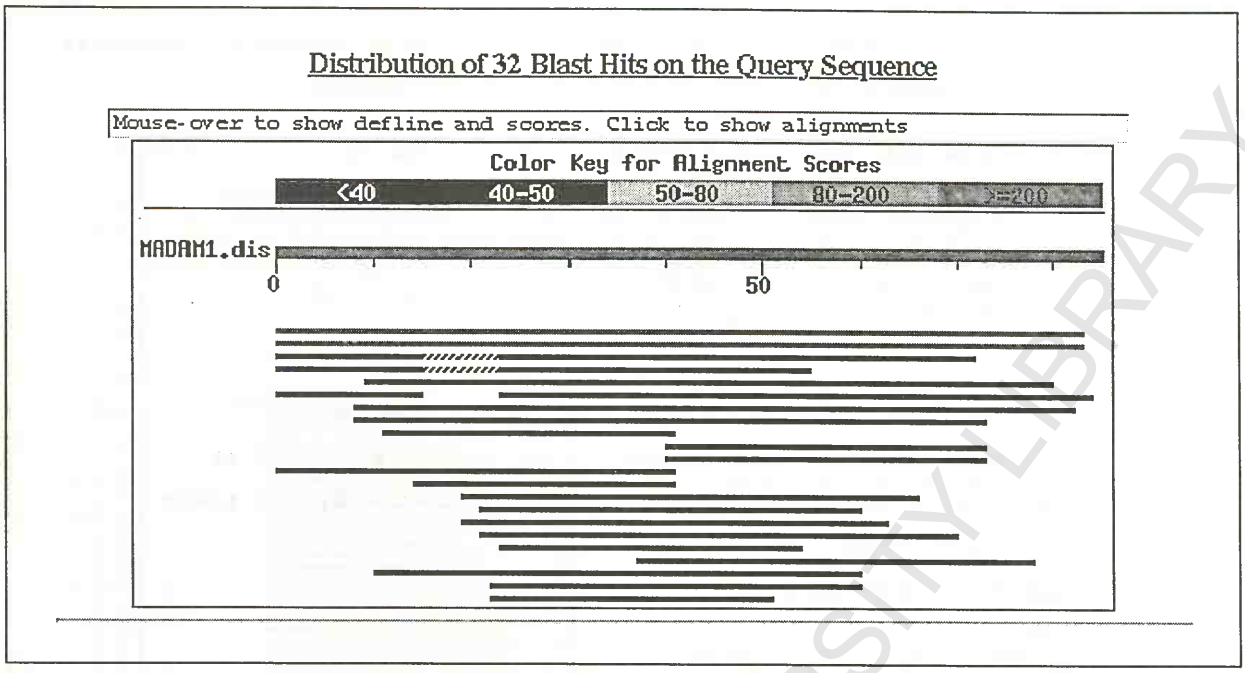

Figure 2.5.9 Example of the graphical view of a TBLASTN report.

Sequences producing significant alignments:	Score (bits)	E Value
emb|Z98857|CEY111B2 Caenorhabditis elegans DNA *** SEQUENCING I...	94	2e-19
emb|Z92819|CEY37D8 Caenorhabditis elegans DNA *** SEQUENCING IN...	94	2e-19
gb|AC005075|AC005075 Homo sapiens clone RG219E16; HTGS phase 1,...	37	0.027
gb|AC002557|AC002557 *** SEQUENCING IN PROGRESS *** Homo sapien...	36	0.035
gb|AC003656|AC003656 *** SEQUENCING IN PROGRESS *** EPM1/APECED...	34	0.23
gb|AC005180|AC005180 *** SEQUENCING IN PROGRESS *** Homo sapien...	33	0.40
gb|AC004877|AC004877 Homo sapiens clone DJ0751H13; HTGS phase 1...	32	0.68
gb|AC003066|AC003066 *** SEQUENCING IN PROGRESS *** Mus musculu...	31	1.2
gb|AC002042|AC002042 *** SEQUENCING IN PROGRESS *** Homo sapien...	31	1.5
gb|AC003097|AC003097 *** SEQUENCING IN PROGRESS *** Human chrom..	31	1.5
gb|AC004303|AC004303 *** SEQUENCING IN PROGRESS *** DS06475; D1...	31	1.5
gb|AC003095|AC003095 *** SEQUENCING IN PROGRESS *** Human chrom..	31	1.5
gb|AC000406|HSAC000406 *** SEQUENCING IN PROGRESS *** Human Chr...	30	3.4
emb|AL022343|HS608E8 Human DNA sequence *** SEQUENCING IN PROGR...	30	3.4
emb|AL022100|HS1059H15 Human DNA sequence *** SEQUENCING IN PRO...	30	3.4
emb|Z92863|CEY64G10 Caenorhabditis elegans DNA *** SEQUENCING I...	30	3.4
emb|AL031279|HS163O16 Human DNA sequence *** SEQUENCING IN PROG...	29	5.9
emb|Z97634|HS367G8 Human DNA sequence *** SEQUENCING IN PROGRES...	29	5.9
gb|AC005025|AC005025 Homo sapiens clone GS464G18; HTGS phase 1,...	29	5.9
gb|AC004618|AC004618 *** SEQUENCING IN PROGRESS *** Homo sapien...	29	5.9
gb|AC005086|AC005086 Homo sapiens clone RG305I02; HTGS phase 1,...	29	7.8
gb|AC004169|AC004169 *** SEQUENCING IN PROGRESS *** Homo sapien...	29	7.8
emb|AL008728|HS125N5 Human DNA sequence *** SEQUENCING IN PROGR...	29	7.8

Figure 2.5.10 Example of the hit list from a TBLASTN report.

shows selected pairwise alignments between the query sequence and the hits. For TBLASTN searches, the top line (Query) of the alignment contains protein sequence, and the location refers to amino acids. The bottom line (Sbjct) of the alignment contains a translated nucleotide sequence, and the location refers to the nucleotide location.

Disintegrin-like domains contain a characteristic pattern of cysteines, so it is usually easy to identify the true hits by checking for conservation in the number of cysteine residues, and the spacing between them. The first hit, a 627-kb *C. elegans* clone, aligns with the

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```
emb|Z98857|CEY111B2 Caenorhabditis elegans DNA *** SEQUENCING IN PROGRESS *** from clone
            Y111B2; HTGS phase 1 [Caenorhabditis elegans]
            Length = 627200

 Score = 94.0 bits (230), Expect = 2e-19
 Identities = 43/84 (51%), Positives = 56/84 (66%), Gaps = 4/84 (4%)

Query: 1       CGNGVVEDLEECDCGS--DCDSHPCCSP-TCTLKEGAQCS-EGLCCYNCTFKKKGSLCRP 56
                CGNGVV+  EECDCGS  +C   PCC P TCTL+  AQC+    CC+ C  +K G  CR
Sbjct: 322651 CGNGVVDGSEECDCGSRENCQD-PCCDPLTCTLRPHAQCAAHHKCCHRCELRKAGDTCRS 322827

Query: 57      AEDVCDLPEYCDGSTQECPANSIMQDGT 84
                ++  CD+ E CDG + +CP +  + DGT
Sbjct: 322828 SKSPCDVAEQCDGKSGDCPPDGHLIDGT 322911


 Score = 46.1 bits (107), Expect = 1e-06
 Identities = 30/55 (54%), Positives = 36/55 (64%), Gaps = 21/55 (38%)

Query: 1       CGNGVVEDLEECDCG---SDCD--SHPCCSP----------TCTLKEGAQC--SEGLCCY 43
                CGN + E  EECDCG   +DCD     CC P           C  K GAQC  S+G CC
Sbjct: 102880 CGNQIYEPGEECDCGFSQADCDQMGDKCCVPHEARGNGGPGPCKRKPGAQCSPSQGYCCN 103059

Query: 44      --NCTF--KKKGSLCR 55
                  C+    K +  +CR
Sbjct: 103060 PDTCSLHGKNEEKICR 103107


 Score = 24.3 bits (51), Expect = 1e-06
 Identities = 9/26 (34%), Positives = 12/26 (45%)

Query: 58      EDVCDLPEYCDGSTQECPANSIMQDG 83
                E  C    + CDG   +CP +    DG
Sbjct: 103155 ESECSNLQTCDGRNAQCPVSPPKHDG 103232


 emb|Z92819|CEY37D8 Caenorhabditis elegans DNA *** SEQUENCING IN PROGRESS *** from clone
            Y37D8; HTGS phase 1 [Caenorhabditis elegans]
            Length = 353436

 Score = 94.0 bits (230), Expect = 2e-19
 Identities = 43/84 (51%), Positives = 56/84 (66%), Gaps = 4/84 (4%)

Query: 1       CGNGVVEDLEECDCGS--DCDSHPCCSP-TCTLKEGAQCS-EGLCCYNCTFKKKGSLCRP 56
                CGNGVV+  EECDCGS  +C   PCC P TCTL+  AQC+    CC+ C  +K G  CR
Sbjct: 304601 CGNGVVDGSEECDCGSRENCQD-PCCDPLTCTLRPHAQCAAHHKCCHRCELRKAGDTCRS 304425

Query: 57      AEDVCDLPEYCDGSTQECPANSIMQDGT 84
                ++  CD+ E CDG + +CP +  + DGT
Sbjct: 304424 SKSPCDVAEQCDGKSGDCPPDGHLIDGT 304341


 gb|AC005075|AC005075 Homo sapiens clone RG219E16; HTGS phase 1, 3 unordered pieces [Homo
            sapiens]
            Length = 198776

 Score = 36.7 bits (83), Expect = 0.027
 Identities = 13/26 (50%), Positives = 16/26 (61%)

Query: 24      CSPTCTLKEGAQCSEGLCCYNCTFKK 49
                C   CTL + +QCS+GLCC  C   K
Sbjct: 53474 CCKKCTLTQDSQCSDGLCCKKCKVNK 53551


 Score = 31.7 bits (70), Expect = 0.89
 Identities = 11/16 (68%), Positives = 13/16 (80%)

Query: 1       CGNGVVEDLEECDCGS 16
                CGNG +E  EECDCG+
Sbjct: 53178 CGNGFIETGEECDCGT 53225


 Score = 29.0 bits (63), Expect = 5.9
 Identities = 10/27 (37%), Positives = 18/27 (66%)

Query: 47      FKKKGSLCRPAEDVCDLPEYCDGSTQE 73
                F+  G++CR A + CD+ E C G++ +
Sbjct: 57012 FQPMGTVCREAVNDCDIRETCSGNSSQ 57092
```

Figure 2.5.11 Example of a TBLASTN alignment.

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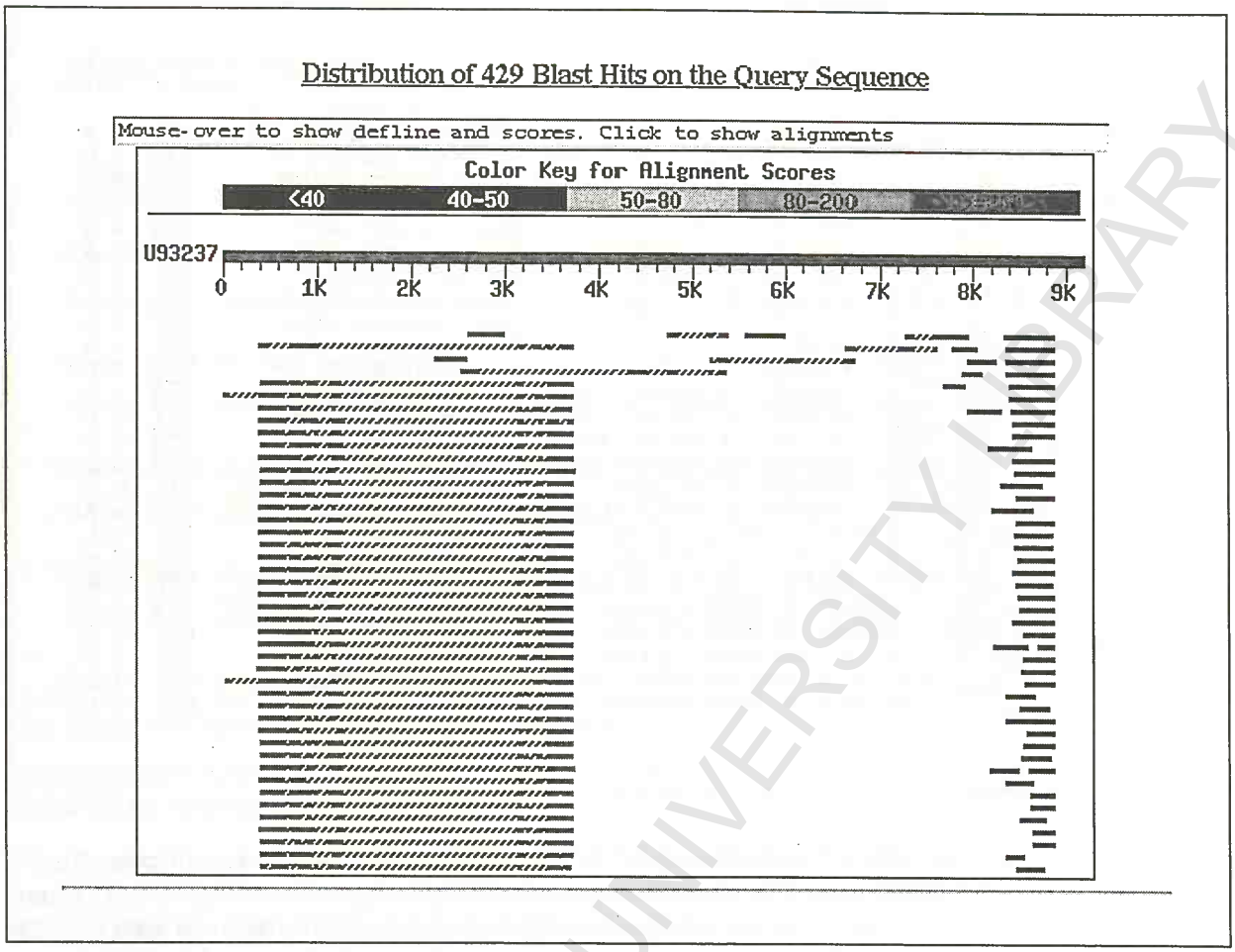

Figure 2.5.12 Example of the graphical view of a BLASTN report.

query sequence in two regions. Both alignments encompass the full length of the query sequence, and both contain a characteristic alignment of cysteine residues. The first region (the first aligned block) comprises nucleotides 322651-322911 of the *C. elegans* clone. The second region consists of the second and third alignment blocks. The first 55 amino acids of the disintegrin domain align with nucleotides 102880-103107 of the clone, and the last 25 amino acids align with nucleotides 103155-103232 of the clone. Thus, this *C. elegans* clone appears to encode two distinct disintegrin-like domains, and these two sequences are likely linked in the *C. elegans* genome. The region between nucleotides 103107-103155 of the clone may contain an intron. The second database hit is to a shorter *C. elegans* clone that, from the sequence of its encoded disintegrin domain, appears to contain some of the same sequence as the first *C. elegans* clone. The third database hit is to a human clone. As amino acids 1 to 16 of the query aligns with nucleotides 53178 to 53225 of the hit, and amino acids 24 to 49 align with nucleotides 53474 to 53551, the human sequence may contain an intron between nucleotides 53225 and 53474. There is a likely second intron between nucleotides 53551 and 57012.

BLASTN

Figure 2.5.12 presents the graphical overview of a BLASTN search of accession number U93237 against the human EST database. U93237 is the 9.2-kb genomic sequence of the human menin (MEN1) gene for multiple endocrine neoplasia type I. This example demonstrates the power of a BLAST search to confirm exons in a genomic sequence, as well as the danger of accepting results without further investigation. The matches on the

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```
                                                                  Score    E
Sequences producing significant alignments:                       (bits)  Value

gb|AA776738|AA776738  ah49f08.s1 Soares testis NHT Homo sapiens ...   950  0.0
gb|AI082242|AI082242  ox79g05.x1 Soares_NhHMPu_S1 Homo sapiens c...   942  0.0
gb|AA743431|AA743431  ny23f01.s1 NCI_CGAP_GCB1 Homo sapiens cDNA...   940  0.0
gb|AA705195|AA705195  zj96e04.s1 Soares fetal liver spleen 1NFLS...   922  0.0
gb|AA973790|AA973790  oo45g04.s1 NCI_CGAP_Lu5 Homo sapiens cDNA ...   862  0.0
gb|AA604513|AA604513  no73c10.s1 NCI_CGAP_AA1 Homo sapiens cDNA ...   850  0.0
emb|Z98467|HSZ98467  Homo sapiens mRNA; expressed sequence tag; ...   841  0.0
gb|AI056630|AI056630  oz18e01.x1 Soares_fetal_liver_spleen_1NFLS...   809  0.0
gb|AA636030|AA636030  nr37h07.s1 NCI_CGAP_Pr22 Homo sapiens cDNA...   797  0.0
gb|AA614240|AA614240  np09f12.s1 NCI_CGAP_Pr3 Homo sapiens cDNA ...   791  0.0
gb|AA147620|AA147620  zl52d02.s1 Soares pregnant uterus NbHPU Ho...   777  0.0
gb|AA262936|AA262936  zr71a02.s1 Soares NhHMPu S1 Homo sapiens c...   773  0.0
gb|AA147612|AA147612  zl52d02.r1 Soares pregnant uterus NbHPU Ho...   761  0.0
gb|AI078460|AI078460  oz13e02.x1 Soares_fetal_liver_spleen_1NFLS...   755  0.0
gb|AA209475|AA209475  zq84c11.s1 Stratagene hNT neuron (#937233)...   747  0.0
gb|AA459317|AA459317  aa25e05.r1 NCI_CGAP_GCB1 Homo sapiens cDNA...   741  0.0
gb|AA805578|AA805578  ob44g06.s1 NCI_CGAP_GCB1 Homo sapiens cDNA...   739  0.0
gb|H41351|H41351  yp71b11.s1 Homo sapiens cDNA clone 192861 3'.      716  0.0
gb|AA157374|AA157374  zo68h09.s1 Stratagene pancreas (#937208) H...   710  0.0
gb|AA775293|AA775293  ad18f11.s1 Soares NbHFB Homo sapiens cDNA ...   706  0.0
gb|H97384|H97384  yw03h05.s1 Homo sapiens cDNA clone 251193 3'.      702  0.0
gb|H91639|H91639  yv03g12.s1 Homo sapiens cDNA clone 241702 3'.      666  0.0
gb|N51774|N51774  yz01g01.s1 Homo sapiens cDNA clone 281808 3'.      666  0.0
emb|Z98466|HSZ98466  Homo sapiens mRNA; expressed sequence tag; ...   654  0.0
gb|H38333|H38333  yp70g02.r1 Homo sapiens cDNA clone 192818 5'.      636  e-180
```

Figure 2.5.13 Example of the hit list from a BLASTN report.

right half of the diagram (base pairs 4000 to 9000) confirm the locations of exons 2 to 10 of the MEN1 gene (Chandrasekharappa et al., 1997; Zhang and Madden, 1997). The large number of matches on the left side of the figure (base pairs 500 to 1000 and 3000 to 3600) appear at first glance to be interesting matches, but are actually caused by the presence of *Alu* elements in the query and EST sequences. One can determine this by checking the definition lines of the matches in this region, as many of these ESTs are annotated as being similar to *Alu* elements. It is important to remember, though, that the definition line summaries (e.g., Fig. 2.5.13) contain truncated definition lines, and that it is necessary to look at the full definition line (provided in the alignment section, Fig. 2.5.14) to see the *Alu* annotation. One can also perform a BLASTN search of U93237 against the Alu database.

Fig. 2.5.13 shows the top portion of the list of database hits for the BLASTN search of U93237 against human ESTs. Fig. 2.5.14 presents one alignment of the query sequence with an EST containing an *Alu* repeat. Vertical bars connect bases that are identical in the query and subject. Since both the query and the subject are nucleotide sequences, the numbers shown refer to nucleotide sequence locations. Nucleotides in the query sequence that have been masked for low complexity are replaced by the letter n (e.g., nnnnnnn in the third block). BLASTN checks the plus and minus strand of the query against the plus strand of the database sequence. The alignment shown here presents the plus strand of the query to the minus strand of the database sequence, which is equivalent to showing the minus strand of the query against the plus strand of the database sequence.

BLASTN is optimized for speed, not sensitivity. The algorithm uses a simple system to assess the alignments; matches are given a positive score, mismatches a negative score. BLASTN should only be used when a direct comparison of nucleotide sequences is desired. BLASTN results should not be used to make any predictions about the functions of the encoded proteins. Generally, searches that involve protein comparisons are more sensitive, owing to the larger alphabet (twenty residues versus four nucleotides) and the

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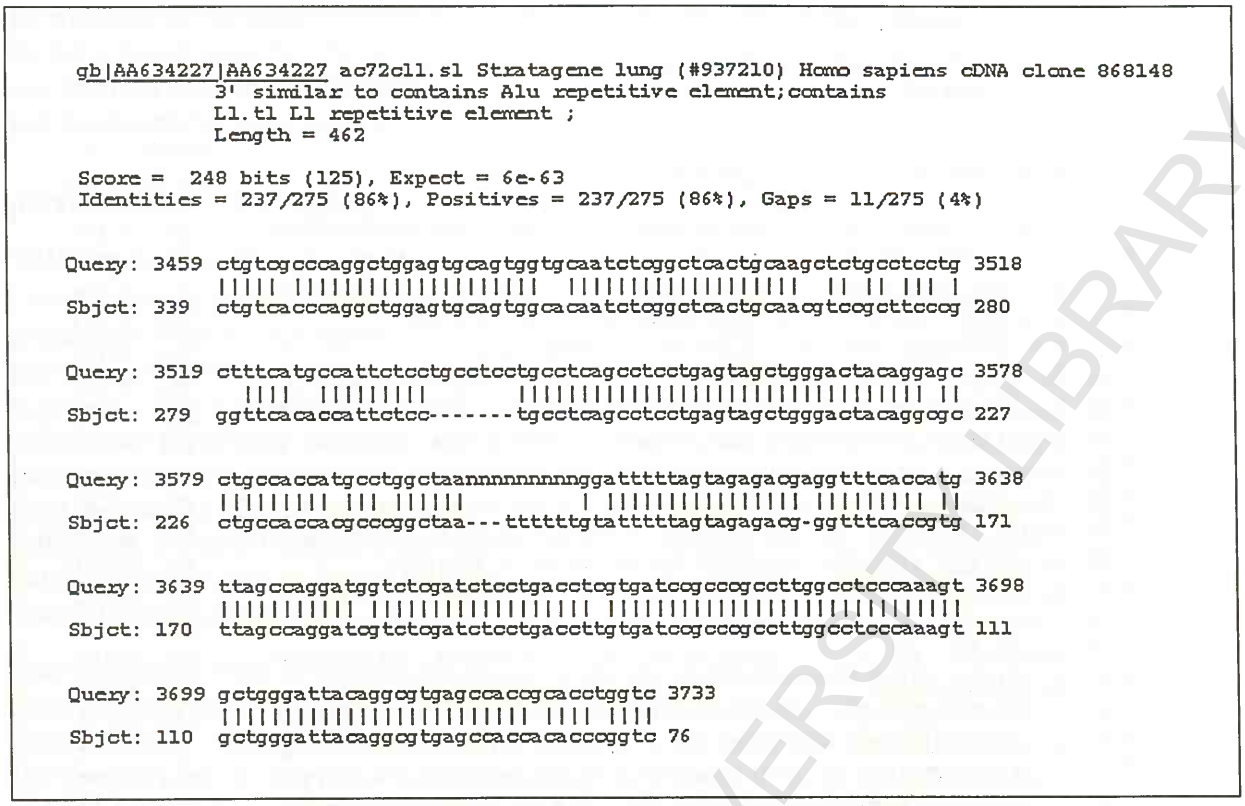

Figure 2.5.14 Example of a BLASTN alignment.

degeneracy of the genetic code (two codons, with different third bases often coding for the same protein). The more sophisticated scoring system used with protein comparisons also takes into account similarities between different residues.

PSI-BLAST

A major new feature of the BLAST version 2.0 is the iterative search. Position-specific iterated BLAST (PSI-BLAST) first performs a normal BLASTP run, but then internally computes a profile using the most significant hits (Altschul et al., 1997). A profile may be understood as a table that lists the probability of finding a residue at each position in a conserved protein domain. Conserved regions between the query and the most-significant matches are used to compute the profile. These regions are presumably important to the function of the protein. The profile is then used to perform another search (or iteration), at which point the new hits are also added into the profile. The cycle may then be repeated, with a new profile computed from the results of the last iteration. Each PSI-BLAST iteration takes into account the most significant sequences found in the previous round (i.e., those below the threshold for inclusion in the profile). The algorithm is more sensitive than BLASTP, and more likely to lead to the discovery of distantly related sequences. At present, such iterative searching is only available with the BLASTP program.

The PSI-BLAST output is different from the other programs. Figure 2.5.15 shows the one-line summaries from the first PSI-BLAST iteration (i.e., the first search using the profile) of a mouse adenosine deaminase (Swiss-Prot accession P03958) against the nr database. The one-line summaries are divided into two groups, those with an *E* value better (lower) than that used to build the profile (Fig. 2.5.15; the default value is 0.001), and those with a worse *E* value (not shown). Matches better than the threshold, which will be included in the profile, are checked. The user may check (or uncheck) matches, causing them to be included (or excluded) from the profile used in the next iteration. Matches found on a previous iteration are marked with a green ball. New sequences are

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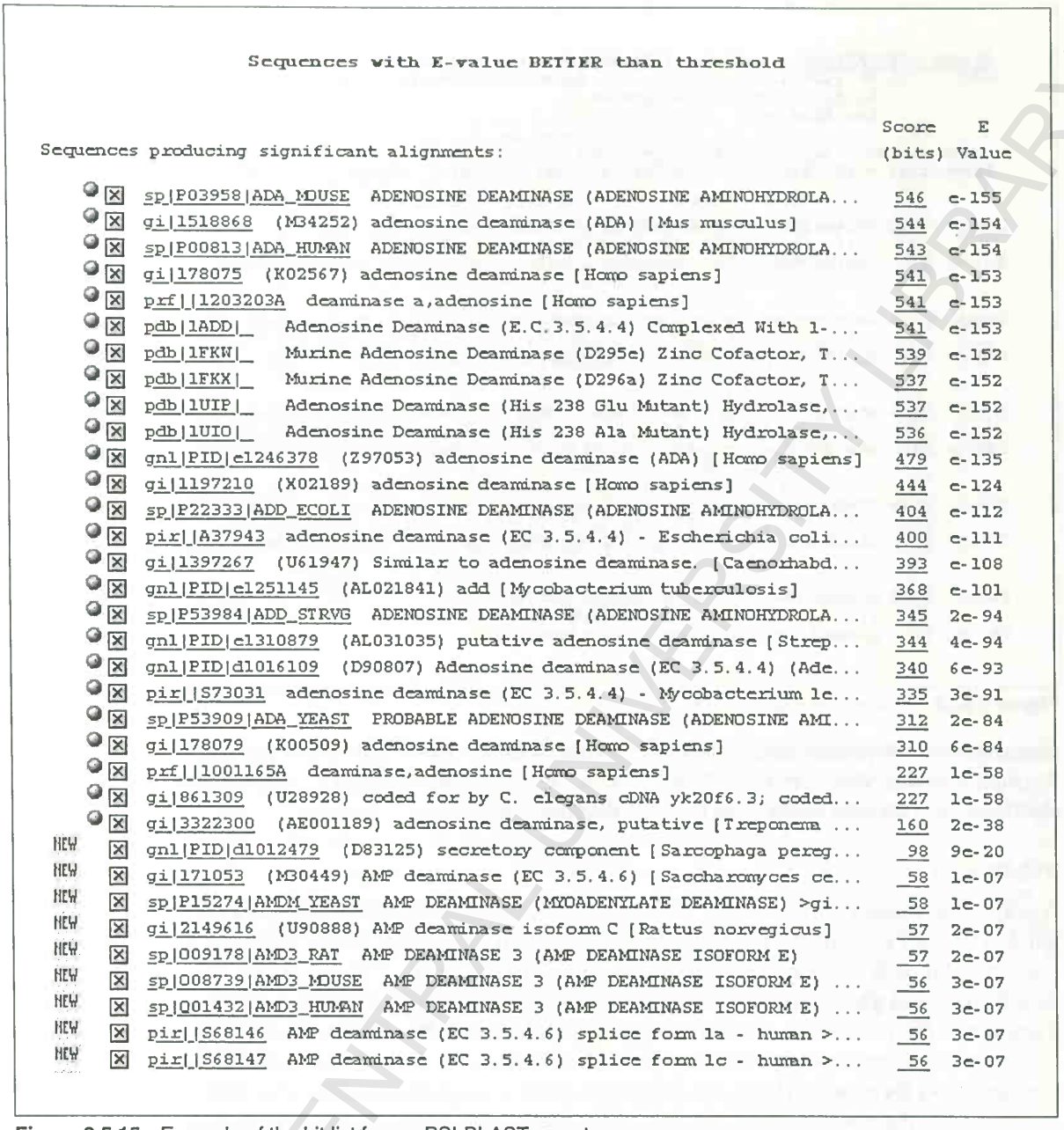

Figure 2.5.15 Example of the hit list from a PSI-BLAST report.

marked with a yellow "new". The page also alerts the user when convergence is achieved (i.e., no new sequences below the threshold for inclusion in the profile were found in that iteration).

In the hit list in Figure 2.5.15, the first iteration of PSI-BLAST finds many adenosine deaminase sequences (marked with a green ball) found in the previous (standard) BLASTP search. In addition, PSI-BLAST identifies a number of AMP deaminase sequences (marked with "new"). Indeed, there is a well-known homology between adenosine deaminase and AMP deaminase (Chang et al., 1991; Holm and Sander, 1997) that would not normally be found by BLASTP, but is found by PSI-BLAST.

If one wants to report the *E* value for a hit found with a PSI-BLAST search, one should use the *E* value calculated during the first iteration that the match is below the threshold

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for inclusion in the profile (i.e., when the hit is marked with a "new" flag). However, if the hit is found in the first BLASTP run, then the reported *E* value should be from this run. In either case, in subsequent iterations, the hit itself is used to compute the profile, and the reported *E* value is not representative of the true value.

SEARCHING STRATEGIES

Filtering for Low-Complexity Regions

Low-complexity regions tend to result in misleading database search results. The residue frequencies within such regions differ dramatically from the database as a whole, disrupting the statistics used by BLAST. This can produce alignments that are based purely on compositional bias rather than a significant position-by-position alignment. By default, low-complexity sequences are filtered out of query sequences and replaced by strings of n (nucleotide) or X (protein). A BLASTP search of a proline-rich, DNA-directed RNA polymerase (Swiss-Prot accession P11414) against the nr database illustrates the differences in output resulting from a filtered or unfiltered query sequence. Twenty four database matches with an *E* value better than 10 are found if filtering is enabled; 4873 are found if filtering is disabled.

The top-scoring database matches for the unfiltered search are shown in Figure 2.5.16; high-scoring matches that are not found in a filtered search are marked. Note that the definition lines of most of the new matches indicate that the sequences are proline-rich, like the query, but do not provide information about the function of the protein. Other hits to RNA polymerases, found near the top of the list in a filtered search, are buried much deeper in the list of matches. The match to "splP14248," apparently a good match based on the definition line, does not appear in a filtered BLAST search. An examination of the alignment (not shown) indicates that the match is based on solely on proline-rich regions.

Reporting BLAST Results

The most reliable indicator of the importance of a BLAST alignment is the Expect (*E*) value (the number of chance database matches one expects to see at the same score). This number takes into account the length of the query and size of the database as well as the scoring system. Neither the bit nor the raw score is a reliable indicator, as their significance cannot be judged independently of the information used to calculate the *E* value. Normal intuition fails when one is faced with databases of the current size (e.g., the EST database contains ~700 million base pairs at this time), as chance alignments are likely and it is impossible for a user to know at what score matches become significant. Statistical significance also varies from database to database (e.g., a hit with a certain score may be statistically significant in a search of the relatively small month database, but not in a search of nr), so that reporting a certain score without the context of the database size can be misleading. The percentage identity is also a poor indicator of statistical significance for the same reasons; additionally, there is really no way to estimate an *E* value or even a score from a percentage identity, making it impossible to even guess whether a hit could be due to chance or not.

Analyzing mRNA Sequences

1. Identify the open reading frame to obtain the protein sequence. Perform a BLASTX search of the cDNA sequence against the nr protein database. Any resulting hits may identify the correct reading frame for the protein sequence and provide information about its function.

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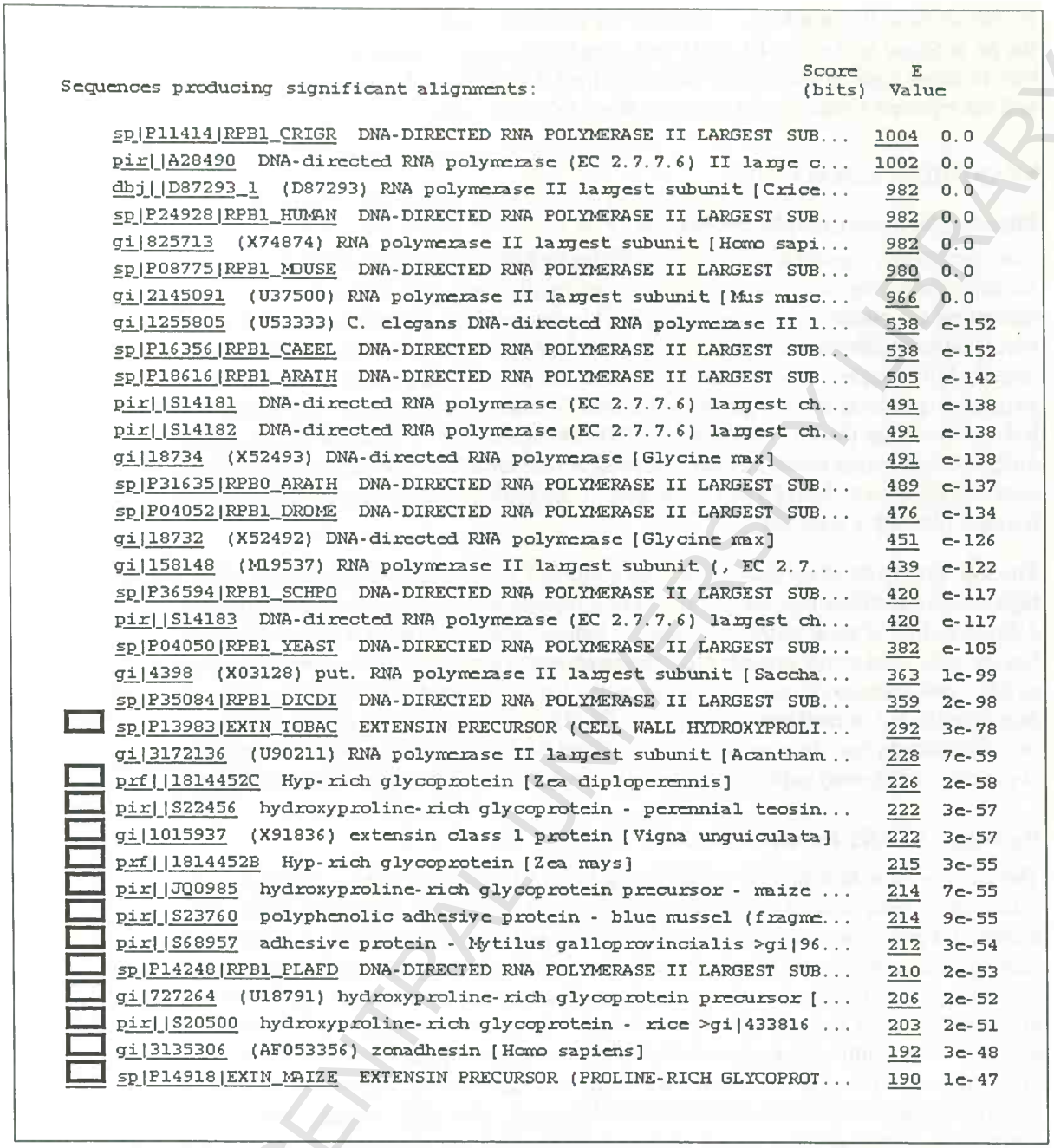

Sequences producing significant alignments:	Score (bits)	E Value
sp|P11414|RPB1_CRIGR DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	1004	0.0
pir||A28490 DNA-directed RNA polymerase (EC 2.7.7.6) II large c...	1002	0.0
dbj||D87293_1 (D87293) RNA polymerase II largest subunit [Cricc...	982	0.0
sp|P24928|RPB1_HUMAN DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	982	0.0
gi|825713 (X74874) RNA polymerase II largest subunit [Homo sapi...	982	0.0
sp|P08775|RPB1_MOUSE DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	980	0.0
gi|2145091 (U37500) RNA polymerase II largest subunit [Mus musc...	966	0.0
gi|1255805 (U53333) C. elegans DNA-directed RNA polymerase II 1...	538	e-152
sp|P16356|RPB1_CAEEL DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	538	e-152
sp|P18616|RPB1_ARATH DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	505	e-142
pir||S14181 DNA-directed RNA polymerase (EC 2.7.7.6) largest ch...	491	e-138
pir||S14182 DNA-directed RNA polymerase (EC 2.7.7.6) largest ch...	491	e-138
gi|18734 (X52493) DNA-directed RNA polymerase [Glycine max]	491	e-138
sp|P31635|RPB0_ARATH DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	489	e-137
sp|P04052|RPB1_DROME DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	476	e-134
gi|18732 (X52492) DNA-directed RNA polymerase [Glycine max]	451	e-126
gi|158148 (M19537) RNA polymerase II largest subunit (, EC 2.7....	439	e-122
sp|P36594|RPB1_SCHPO DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	420	e-117
pir||S14183 DNA-directed RNA polymerase (EC 2.7.7.6) largest ch...	420	e-117
sp|P04050|RPB1_YEAST DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	382	e-105
gi|4398 (X03128) put. RNA polymerase II largest subunit [Saccha...	363	1e-99
sp|P35084|RPB1_DICDI DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	359	2e-98
sp|P13983|EXTN_TOBAC EXTENSIN PRECURSOR (CELL WALL HYDROXYPROLI...	292	3e-78
gi|3172136 (U90211) RNA polymerase II largest subunit [Acantham..	228	7e-59
prf||1814452C Hyp-rich glycoprotein [Zea diploperennis]	226	2e-58
pir||S22456 hydroxyproline-rich glycoprotein - perennial teosin...	222	3e-57
gi|1015937 (X91836) extensin class 1 protein [Vigna unguiculata]	222	3e-57
prf||1814452B Hyp-rich glycoprotein [Zea mays]	215	3e-55
pir||JQ0985 hydroxyproline-rich glycoprotein precursor - maize ...	214	7e-55
pir||S23760 polyphenolic adhesive protein - blue mussel (fragme...	214	9e-55
pir||S68957 adhesive protein - Mytilus galloprovincialis >gi|96...	212	3e-54
sp|P14248|RPB1_PLAFD DNA-DIRECTED RNA POLYMERASE II LARGEST SUB...	210	2e-53
gi|727264 (U18791) hydroxyproline-rich glycoprotein precursor [...	206	2e-52
pir||S20500 hydroxyproline-rich glycoprotein - rice >gi|433816 ...	203	2e-51
gi|3135306 (AF053356) zonadhesin [Homo sapiens]	192	3e-48
sp|P14918|EXTN_MAIZE EXTENSIN PRECURSOR (PROLINE-RICH GLYCOPROT...	190	1e-47

Figure 2.5.16 Example of a hit list from a BLASTP report in which the query sequence was not filtered. Black squares, added manually by the authors, indicate hits that would not appear if the query had been filtered.

2. Determine whether the protein sequence is similar to that of any previously identified proteins. Perform a BLASTP and a PSI-BLAST (iterative) search against the nr protein database using the protein sequence as a query.

3. Determine whether the protein sequence is similar to the translation products of any uncharacterized DNA sequences whose translations are not in nr. Perform a TBLASTN search of the EST, STS, GSS, and HTGS databases.

4. After the initial search, perform a monthly search of the month nucleotide and protein sequence databases to provide information about newly added sequences. A BLASTP

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search of the month protein database will reveal newly characterized protein sequences. A TBLASTN search of the month nucleotide database will reveal new open reading frames in nr, EST, HTGS, STS, and GSS. PSI-BLAST searches of nr should also be conducted occasionally, as new sequences may be added to the profile that will, in turn, help identify more distantly related sequences.

5. If the protein sequence contains multiple functional domains, it may be useful to perform the searches with each of these domains individually.

Analyzing Genomic DNA Sequences

1. Identify a potential transcribed segment. Perform a BLASTN search of the nucleotide sequence against the DNA sequences in the EST database to determine if any mRNAs are derived from the genomic sequence. EST hits will highlight the location of exons. A BLASTN search against nr may identify transcripts or previously characterized genomic segments.
2. Identify potential open reading frames. Perform a BLASTX search against the nr protein database. Any resulting hits may identify the correct reading frame of a protein sequence and provide information about its function. If no results are found with the previous methods, a TBLASTX search (translated nucleotide query versus a translated nucleotide database) against EST, GSS, HTGS, and STS may identify other open reading frames.
3. After the initial search, a monthly search of the month nucleotide (with BLASTN) and protein (with BLASTX) sequence databases will provide information about newly added sequences.
4. Any potential transcribed sequences can be analyzed as per the above instructions for mRNA sequences.

Searching Short Sequences

Short sequences can only produce short alignments. Such short alignments need to be relatively strong (i.e., have a high percentage of matching residues) to rise above the background noise. Short, but strong, alignments are more easily detected using a matrix with a higher relative entropy than that of the default BLOSUM-62 (Altschul, 1991). Relative entropy is basically the average information available per position to distinguish the alignment from chance. The BLOSUM series of matrices does not include any that are suitable for the shortest queries, so it is recommended to use the PAM matrices instead (Dayhoff et al., 1978; Schwartz and Dayhoff, 1978). These matrices may be chosen from a menu on the Advanced BLAST page. Suggested matrices for different query lengths are shown in Table 2.5.2 (S.F. Altschul, pers. comm.).

Table 2.5.2 Substitution Matrices for Short Sequences

Query length (amino acids)	Matrix
<35	PAM-30
35-50	PAM-70
50-85	BLOSUM-80
>85	BLOSUM-62

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SEQUENCE ALIGNMENT ALGORITHMS

There are two types of pairwise sequence alignments, global and local. Global strategies attempt to align along the entire length of both sequences, while local alignments focus on sequence similarity over shorter regions. Historically, local alignments have been very successful at identifying similarities between proteins. Subsequences of similarity, surrounded by unrelated sequences, can identify, for example, active sites that are conserved between proteins or exons in genomic sequence. Smith and Waterman (1981) were the first to develop a local alignment algorithm. However, such rigorous alignment algorithms are often quite slow and impractical for searching current databases on a routine basis.

Heuristic algorithms implement certain shortcuts to allow a comparison against an entire database within a reasonable time. They are not exhaustive searches in the sense that they do not explore possibilities that seem unlikely to be interesting. Some programs allow the user to adjust parameters that increase the sensitivity of the search (i.e., explore more possibilities), but this must be balanced against the slower speed of the search. Studies have shown that heuristic algorithms, with default settings, approach the sensitivity of exhaustive searches (Altschul et al., 1997). BLAST (Altschul et al., 1990, 1997) and FASTA (Pearson, 1990; Pearson and Lipman, 1988) are two popular heuristic programs. The rest of this discussion will focus on the newest version (2.0) of BLAST.

BLAST calculates a statistical significance for every alignment it produces. Significance is expressed in terms of the *E* value, which is the number of matches with a given score (the calculation of the score is described below) that one would expect by chance. The *E* value depends on the size of the database and the length of the query, as increasing either of these parameters increases the number of chance matches. Calculation of the *E* value is performed with Karlin-Altschul statistics (Karlin and Altschul, 1990, 1993).

The raw score of an alignment is calculated with a scoring system, which is a table of residue substitution scores and penalties for the existence and extension of a gap. For normal protein/protein comparisons, a matrix (i.e., table of residue substitution scores) provides a score for each possible alignment of two amino acids. For example, L aligned with I gets a positive score, while L aligned with E gets a negative score. BLOSUM-62 is the default matrix, and experimentation has shown that it is among the best for detecting weak protein similarities (Henikoff and Henikoff, 1992). PSI-BLAST uses a table that lists the frequency of finding a certain residue at each position in a conserved protein domain. This table is calculated "on the fly" for each iteration from the best matches to the database. BLASTN uses a simple scheme where matches have a positive score and mismatches have a negative score. Gaps are opened or extended when one sequence is longer than another over a certain region. BLAST charges the score $-a$ for the existence of a gap and $-b$ for each letter in a gap, so that a gap of length k letters is penalized $-(a + bk)$. Gap costs of this form are known as affine gap costs. These gap costs may be changed by the user.

The scoring system determines the raw score of an alignment. A bit score can be calculated from the raw score using the following formula:

$$S_{bit} = [\lambda \times S_{raw} - \ln(K)]/\ln(2)$$

where λ and K are Karlin-Altschul parameters that depend on the scoring system. The statistical significance corresponding to the resulting bit score is independent of the scoring system used. This allows one to compare the bit scores obtained from two different BLAST runs, each performed using different matrices or gap extension and existence values. Nevertheless, it is more meaningful to compare the results of different BLAST

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runs using their *E* values rather than their bit scores, as the *E* value takes into account the size of the database.

A word-based approach is used to find matches in BLAST. An ungapped search, with BLASTN, may be used to demonstrate the concept of a word. First a list is made of all the subsequences in the nucleic acid query sequence that are of a certain length (called the word size), and the positions are recorded. The database is then scanned, looking for words that are in the list. If a match is found between a query and a database word, the alignment is extended (without allowing gaps) until the corresponding score drops a certain amount below the maximum found, at which point that maximum score (and the corresponding alignment) is used. The aligned region is a local alignment. With BLASTN, only exact matches are extended and the default word size is 11 bp.

The procedure is more complicated for the other programs (BLASTP, BLASTX, TBLASTN, and TBLASTX). Exact word matches are not required and the default word size is three residues. A match between a query word and a database word is based on the scoring matrix, which specifies whether two residues result in a positive, negative, or neutral match. If three residues in a query word are compared with three residues in a database sequence, and the score is above a certain threshold, this word warrants further consideration. The default behavior in BLAST version 2.0 is that two words (on the query) must match with two words (on the database sequence) within a window of 40 residues before the match is extended, as described for BLASTN. The effect of looking for two word matches is to eliminate random matches, increasing the speed of BLAST by a factor of three.

Ungapped alignments are used as a starting point for the gapped alignments that BLAST produces. The eleven highest-scoring letters are determined and the center letter is used as the start of the gapped alignment. The alignment is then extended, with gaps allowed. This extension is not exhaustive; if the score drops by more than a certain amount from the maximum found so far, that maximum (and the corresponding alignment) is used (Altschul et al., 1997; Zhang et al., 1998).

The default BLAST parameters are tuned for a balance of speed and sensitivity. It is possible that significant matches may be missed, especially if small databases are searched. In that case, it may be advisable to use a shorter word size for BLASTN or a lower-threshold word for the other programs.

It is important to remember that BLAST calculates similarity. Homology is an evolutionary relationship that must be proved by further analysis.

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APPENDIX A: BLAST PARAMETERS

The Advanced BLAST page offers the possibility to change a number of BLAST parameters. The most important options supported are listed below.

Descriptions (pull-down menu) restricts the number of one-line descriptions to the number specified. The default is 500.

Alignments (pull-down menu) restricts the number of database hits for which alignments are shown. Several alignments may be associated with one database sequence, so the number of alignments may actually be larger than this number. The default is 500.

Expect value (pull-down menu; also known as the *E* value) is the statistical significance threshold for reporting matches. The default value is 10, such that ten alignments are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). Lower *E* thresholds are more stringent, leading to fewer chance matches being reported. Fractional values are acceptable.

Filtering (pull-down menu) masks off segments of the query sequence that have low compositional complexity, as determined by the SEG program (Wootton and Federhen, 1993, 1996) or, for BLASTN, by the DUST program (R.L. Tatusov and D.J. Lipman, pers. comm.). Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other programs.

Genetic code (pull-down menu) selects the genetic code to be used for a BLASTX translation of the query. The default setting is the universal code. The choice of code is determined by the source of the query sequence (e.g., species, mitochondrial).

Graphical overview (checkbox) selects whether an overview of the database sequences aligned to the query sequence will be shown. The score of an alignment is indicated by one of five different colors, which divides the range of scores into five groups. If more than one alignment is displayed for a database sequence, the two alignments are connected by a cross-hatched bar. Mousing over a match causes the definition and score to be shown in the window at the top; clicking on a bar takes the user to the associated alignments. The overview is on by default.

Matrix (menu) allows one to change the matrix from the default of BLOSUM-62. This is useful for searches against short sequences (see Searching Short Sequences). It is also possible to change the gap existence and extension penalties, but the defaults for a given matrix are recommended.

Organism (pull-down menu) limits the search by organism. The menu provides a list of the most common organisms; others may be entered in a text box.

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APPENDIX B: SEQUENCE IDENTIFIER SYNTAX

The syntax of sequence header lines used by the NCBI BLAST server depends on the database from which each sequence was obtained. Table 2.5.3 lists the identifiers for the databases from which the sequences were derived. For example, an identifier might be *gb|M73307|AGMA13GT*, where the gb tag indicates that the identifier refers to a GenBank sequence, M73307 is its GenBank accession number, and AGMA13GT is its GenBank locus.

NCBI assigns gi identifiers for all sequences contained within NCBI's sequence databases (Ostell and Kans, 1998). The gi identifier provides a uniform and stable naming convention whereby a specific sequence is assigned its unique gi identifier. If a nucleotide or protein sequence changes, a new gi identifier is assigned, even if the accession number of the record remains unchanged. Thus, gi identifiers provide a mechanism for identifying the exact sequence that was used or retrieved in a given search.

For searches of the nr protein database where the sequences are derived from conceptual translations of sequences from the nucleotide databases the gi syntax is *gi|gi_identifier*. An example would be *gi|451623 (U04987) env [Simian immunodeficiency...*, where 451623 is the gi identifier and U04987 is the accession number of the nucleotide sequence from which it was derived.

Users may select the *-gi* option for BLAST output, which will produce a header line with the gi identifier concatenated with the database identifier of the database from which it was derived. For example, *gi|176485|gb|M73307|AGMA13GT* would be used for a match from a nucleotide database, and *gi|129295|sp|P01013|OVAX_CHICK* for a protein database.

The gnl (general) identifier allows databases not listed in Table 2.5.3 to be identified with the same syntax. An example here is the PID identifier *gnl|PID|e1632*. PID stands for Protein-ID, and the e in e1632 indicates that this ID was issued by EMBL. As mentioned above, use of the *-gi* option produces the NCBI gi (in addition to the PID), which users can also use to retrieve sequences of interest.

Table 2.5.3 Identifier Syntax for Sequence Databases

Database	Identifier syntax
GenBank	*gb|accession|locus*
EMBL Data Library	*emb|accession|locus*
DDBJ (DNA Data Bank of Japan)	*dbj|accession|locus*
NBRF PIR	*pir||entry*
Protein Research Foundation	*prf||name*
Swiss-Prot	*sp|accession|entry name*
Brookhaven Protein Data Bank	*pdb|entry|chain*
Patents	*pat|country|number*
GenInfo Backbone Id	*bbs|number*
General database identifier	*gnl|database|identifier*

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Ion-Exchange Chromatography

Ion-exchange chromatography separates biomolecules on the basis of charge characteristics. Charged groups on the surface of a protein interact with oppositely charged groups immobilized on the ion-exchange medium. As illustrated in Figure 8.2.1, the charge of a protein depends on the pH of its environment (the operating pH). The pH at which the net charge of a protein is zero (i.e., where the number of positive charges equals the number of negative charges) is known as the isoelectric point (pI). When the operating pH is greater than the pI, the protein will have a net negative charge, and should bind to anion-exchange media, which are positively charged. When the operating pH is less than the pI, the protein will have a net positive charge, and should bind to cation-exchange media, which are negatively charged.

The Strategic Planning section outlines the basic steps in planning and carrying out ion-exchange chromatography to separate proteins. Basic Protocol 1 describes batch adsorption of protein to an ion-exchange medium followed by elution using a step gradient of increasing salt concentration. This technique accommodates a wide range of sample volumes and is most often used in the initial capture stage of protein purification (*UNIT 8.1*). Batch techniques have minimal system requirements. The Alternate Protocol describes use of a buffer of a different pH to elute via step gradient. Basic Protocol 2 describes adsorption of protein to an ion-exchange medium in a column, followed by elution with a linear gradient. Basic Protocol 2 provides a higher resolution than Basic Protocol 1, and is therefore used in the intermediate purification and final polishing stages of protein separation (*UNIT 8.1*). Support Protocol 1 describes a pilot experiment to determine initial conditions for batch or column chromatography (i.e., pH required for binding, change in pH or salt concentration required for elution, and available capacity of a medium). Support Protocol 2 describes a means of calculating the dynamic capacity (*UNIT 8.1*) of an ion-exchange column, Support Protocol 3 describes methods for producing continuous gradients of pH and salt concentration to elute proteins from ion-exchange

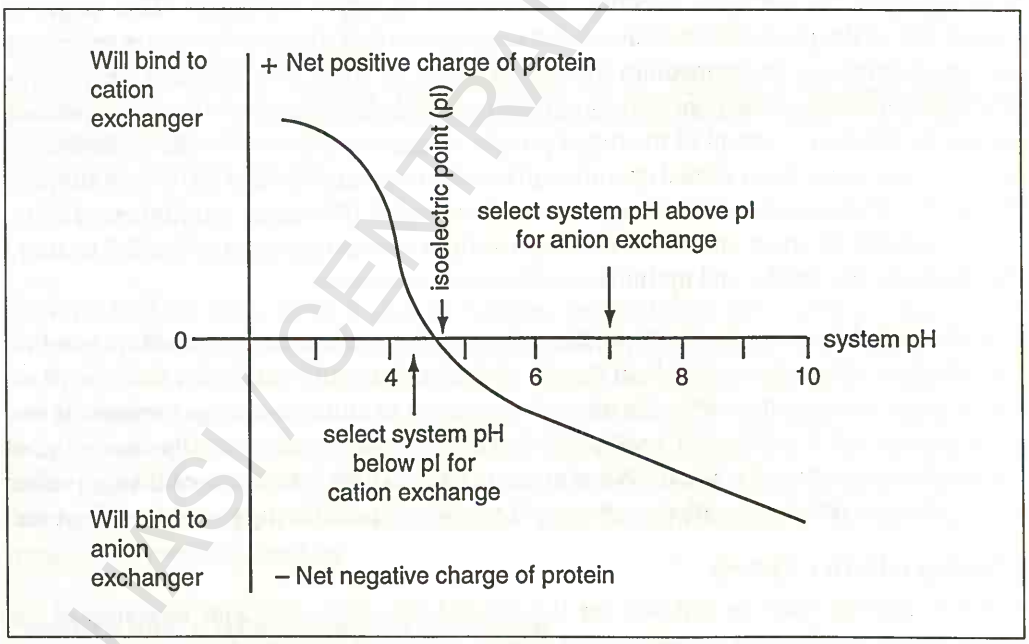

Figure 8.2.1 Net charge of a protein as a function of pH, showing the pH ranges in which protein is bound to anion or cation exchangers. The pH range over which the protein is stable may be only a small fraction of the binding range; this must also be taken into consideration when choosing an ion-exchange medium.

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columns, Support Protocol 4 describes regeneration of used ion-exchange media, and Support Protocol 5 details storage of ion-exchange media.

STRATEGIC PLANNING

There are a number of basic steps in developing an ion-exchange method for protein purification. First, an appropriate ion-exchange medium must be selected, as well as the optimal operating pH and buffer system for the medium and sample (see following discussions on Selecting an Ion-Exchange Medium and Selecting a Buffer System). It is then necessary to decide whether batch or column chromatography is appropriate given the purity, protein concentration, and physical characteristics of the sample, the sample volume to be used, and the availability of suitable equipment (see following discussion on Selecting Batch Versus Column Purification). Next, pilot experiments are conducted to determine conditions for binding and eluting the protein (see Support Protocol 1). It is also necessary to make sure that the capacity of the medium is sufficient to isolate the desired quantity of protein (see Support Protocol 1 and Support Protocol 2). When these initial conditions have been determined, the medium and sample are prepared, the sample is bound to the medium, unbound sample components are washed away, and bound sample components are selectively eluted and collected (see Basic Protocol 1, Basic Protocol 2, or Alternate Protocol). The medium may then be cleaned and regenerated (see Support Protocol 5). Finally, results are evaluated and conditions optimized if necessary (see Critical Parameters).

Selecting an Ion-Exchange Medium

A wide variety of ion-exchange media are commercially available, but no miracle medium exists that is best for every protein purification. Criteria for selecting an ion-exchange medium include the specific requirements of the application, the pI and molecular size of the sample components (i.e., target protein and contaminants), and the available equipment (e.g., pumps and columns).

It is necessary to begin by selecting either anion or cation exchange. This requires knowledge of the pI and pH stability of the target protein. If the pI of the target protein is known, an anion-exchange medium with an operating pH above the pI of the target protein or a cation-exchange medium with an operating pH below the pI of the target protein should be selected. If the pI of the target protein is unknown, it is desirable to determine it before beginning. The optimal operating pH can be determined empirically (see Support Protocol 1). Because the pI for most proteins is below pH 7 (Gianazza and Righetti, 1980), it is reasonable to select an anion-exchange medium and an operating pH of 8.5 to start, then evaluate the results and optimize conditions as necessary.

It is also useful to know the pI and binding characteristics of the contaminants present in the protein solution. For example, if the pH of the binding buffer is higher than the pI of a major contaminant, that contaminant will not bind to an anion-exchange medium. If the starting material is crude cell lysate, DNA will be present and generally needs to be removed from the protein. Since DNA is anionic, it binds tightly to anion-exchange media and typically is not eluted with the salt and pH conditions used during protein purification.

Selecting a Buffer System

A buffer system must be selected for the desired pH range. As with selection of an ion-exchange medium, there are several factors that must be considered in selecting a buffer system, including the type of ion exchange to be performed, the pH stability of the sample and the pH range to be used, the required buffering capacity, and, finally, the cost.

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Table 8.2.1 Buffers for Ion-Exchange Chromatography[a]

pK_a (25°C)	pH range	Buffer[b]	Working concentration (mM)	Temperature factor[c]
Anion exchange				
4.75	4.5-5.0	*N*-Methylpiperazine	20	–0.015
5.68	5.0-6.0	Piperazine	20	–0.015
5.96	5.5-6.0	L-Histidine	20	
6.46	5.8-6.4	bis-Tris	20	–0.017
6.80	6.4-7.3	bis-Tris propane	20	
7.76	7.3-7.7	Triethanolamine	20	–0.02
8.06	7.6-8.5	Tris·Cl	20	–0.028
8.52	8.0-8.5	*N*-Methyldiethanolamine	50	–0.028
8.88	8.4-8.8	Diethanolamine	20 (pH 8.4)	–0.025
8.64	8.5-9.0	1,3-Diaminopropane	20	–0.031
9.50	9.0-9.5	Ethanolamine	20	–0.029
9.73	9.5-9.8	Piperazine	20	–0.026
10.47	9.8-10.3	1,3-Diaminopropane	20	–0.026
11.12	10.6-11.6	Piperidine	20	–0.031
Cation exchange				
2.00	1.5-2.5	Maleic acid	20	
2.88	2.4-3.4	Malonic acid	20	
3.13	2.6-3.6	Citric acid	20	–0.0024
3.81	3.6-4.3	Lactic acid	50	
3.75	3.8-4.3	Formic acid	50	+0.0002
4.21	4.3-4.8	Butanedioic acid	50	–0.0018
4.76	4.8-5.2	Acetic acid	50	+0.0002
5.68	5.0-6.0	Malonic acid	50	
7.20	6.7-7.6	Phosphate	50	–0.0028
7.55	7.6-8.2	HEPES	50	–0.0140
8.35	8.2-8.7	BICINE	50	–0.0180

[a]Information from Pharmacia Biotech (1995).

[b]Abbreviations: BICINE, *N,N*-bis[2-hydroxyethylglycine; bis-Tris, bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane; bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]-propane; HEPES, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid].

[c]Change in pK_a per °C (i.e., $\partial pK_a/\partial T$).

Anionic buffers (e.g., acetate and phosphate) are preferred for cation exchange and cationic buffers (e.g., Tris·Cl, ethanolamine, and piperazine) are preferred for anion exchange. It is important to ensure that the buffering ion will have the same charge as the ion exchanger, and hence will not be bound. A constant buffering capacity and pH will thus be maintained during the ion-exchange experiment. Table 8.2.1 lists a variety of buffers useful for anion and cation exchange. Additives to be included in the buffers (e.g., detergents or protease inhibitors) should also carry the same charge as the ion-exchange medium, to preclude binding.

Selecting Batch Versus Column Purification

If the sample volume is large in relation to the size of the pumps and columns available in the laboratory, batch adsorption techniques (see Basic Protocol 1) are appropriate for the capture stage of purification. Batch methods, in which the sample and medium are directly mixed without use of a column, are employed to reduce the sample volume and

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Table 8.2.2 Ion-Exchange Media for Batch Adsorption

Medium	Type	Supplier[a]
Dextran bead matrix		
QAE Sephadex A-25	Strong anion	Amersham Pharmacia Biotech
QAE Sephadex A-50	Strong anion	Amersham Pharmacia Biotech
SP Sephadex C-25	Strong cation	Amersham Pharmacia Biotech
SP Sephadex C-50	Strong cation	Amersham Pharmacia Biotech
Microgranular cellulose matrix		
QA-52	Strong anion	Whatman
QA-53	Strong anion	Whatman
SE-52	Strong cation	Whatman
SE-53	Strong cation	Whatman
Polymer-coated silica matrix		
Accell Plus QMA	Strong anion	Waters
Accell Plus CM	Weak cation	Waters
Agarose bead matrix		
DEAE Bio-Gel A	Weak anion	Bio-Rad
CM Bio-Gel A	Weak cation	Bio-Rad

[a]For addresses and telephone numbers of suppliers, see *SUPPLIERS APPENDIX*.

total protein content prior to column chromatography. Batch techniques are also very useful for capture of the target protein from extremely crude samples (e.g., cell lysates and samples containing particulates and/or aggregates). In batch methods the flow and packing characteristics of the medium are of minor importance, but economy and capacity are of primary importance. Ion-exchange media suitable for batch purification at the capture stage of protein purification are presented in Table 8.2.2.

High resolution is required in the intermediate purification and polishing stages of protein purification, and this can only be achieved using column chromatography. However, higher-than-necessary resolution is frequently traded off for greater throughput (i.e., amount of material processed in a defined time). The packing and flow characteristics of the medium are of primary importance when using column chromatography. Columns packed with smaller beads usually require higher operating pressures than columns packed with larger beads. However, smaller beads offer higher resolution, resulting from the increased efficiency that comes with decreasing bead size. Larger beads are desirable for applications requiring higher throughput. A medium with the smallest bead size that will provide the necessary throughput (and whose operating pressure can be accommodated by available equipment) should be selected. Table 8.2.3 lists a variety of media suitable for column purification at various stages in the purification process. See *SUPPLIERS APPENDIX* for contact information of suppliers of these media, all of whom provide technical support for selection and use of specific media.

Capacity of Ion-Exchange Media

The amount of ion-exchange medium required for a particular application will be determined by the protein capacity of the medium (i.e., the amount of protein that can be bound to the matrix) at the chosen pH. The amount of medium required will in turn determine the size of column required. The capacity of an ion-exchange medium is dependent on a variety of factors, including charge and molecular size of the components in the sample and experimental conditions employed.

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Table 8.2.3 Ion-Exchange Media for Column Purification[a]

Medium[b]	Matrix	Avg. bead diameter (μm)	Use	Form available	Ionic capacity (meq/ml)	Available capacity (mg/ml)	Dynamic capacity (mg/ml)	Conditions for determining dynamic capacity
Amersham Pharmacia Biotech								
Mono Q	PS/DVB	10	I/P	PP	320	—	65	4.5 mg/ml BSA; 50 mM Tris·Cl, pH 8.0; 100 cm/hr; 0.5 × 5–cm column
Mono S	PS/DVB	10	I/P	PP	160	—	75	Human IgG; 100 mM sodium acetate, pH 5.0; 300 cm/hr; 0.5 × 5–cm column
SOURCE 15Q	PS/DVB	15	I/P	PP/BU	ND	—	54	4.5 mg/ml BSA; 50 mM Tris·Cl, pH 8.0; 100 cm/hr; 0.5 × 5–cm column
SOURCE 15S	PS/DVB	15	I/P	PP/BU	ND	—	85	55 mg/ml lysozyme; 20 mM phosphate, pH 6.8; 1 ml/min; 0.63 × 3–cm column
Q Sepharose High Performance	Agarose	34	C/I/P	PP/BU	170	—	108	4.5 mg/ml BSA; 5 mM Tris·Cl, pH 8.0; 100 cm/hr; 0.5 × 5–cm column
SP Sepharose High Performance	Agarose	34	C/I/P	PP/BU	170	—	90	RNase; 100 mM sodium acetate, pH 5.0; 150 cm/hr; 0.5 × 5–cm column
Q Sepharose Fast Flow	Agarose	90	C/I	PP/BU	215	—	120	Human serum albumin; 50 mM Tris·Cl, pH 8.3
SP Sepharose Fast Flow	Agarose	90	C/I	PP/BU	215	—	50	Human IgG; 100 mM sodium acetate, pH 5.0; 300 cm/hr; 0.5 × 5–cm column
SP Sepharose Big Beads	Agarose	200	C	BU	210	—	80	2 mg/ml BSA; 100 mM sodium acetate, pH 5.0; 300 cm/hr
STREAMLINE DEAE	Agarose	200	B/C	BU	205	—	55	BSA; 50 mM Tris·Cl, pH 7.5; 300 cm/hr; 5-cm-i.d. expanded-bed column
STREAMLINE SP	Agarose	200	B/C	BU	170	—	70	Lysozyme; 50 mM phosphate, pH 7.5, 300 cm/hr; 5-cm-i.d. expanded-bed column
Hi-Trap Q[c]								
HiTrap SP[d]								

continued

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Table 8.2.3 Ion-Exchange Media for Column Purification[a], continued

Medium[b]	Matrix	Avg. bead diameter (μm)	Use	Form available	Ionic capacity (meq/ml)	Available capacity (mg/ml)	Dynamic capacity (mg/ml)	Conditions for determining dynamic capacity
Bio-Rad								
Macro-Prep high Q	PM	10	I/P	PP/BU	115	—	20	5 mg/ml BSA; 50 mM Tris·Cl, pH 8.3; 100 cm/hr; 1.5 × 10–cm column
Macro-Prep high S	PM	10	I/P	PP/BU	127	—	50	5 mg/ml human IgG; 20 mM sodium acetate, pH 5.0; 100 cm/hr; 1.5 × 10–cm column
Macro-Prep high Q	PM	50	B/C/I	PP/BU	400	—	40	5 mg/ml BSA; 50 mM Tris·Cl, pH 8.3; 100 cm/hr; 1.5 × 10–cm column
Macro-Prep high S	PM	50	B/C/I	P/B	160	—	60	5 mg/ml human IgG; 20 mM sodium acetate, pH 5.0; 100 cm/hr; 1.5 × 10–cm column
Econo-Pac high Q[e]								
Econo-Pac high S[f]								
TosoHaas								
Toyopearl QAE-550C	PM	100	C/I	PP/BU	330	70[g]	ND	—
Toyopearl SP-550C	PM	100	C/I	PP/BU	160	110[h]	ND	—
Toyopearl SUPER Q-650C	PM	100	C/I	PP/BU	280	129[g]	ND	—
Toyopearl SUPER Q-650M	PM	65	C/I/P	PP/BU	240	143[g]	ND	—
Toyopearl SUPER Q-650S	PM	35	I/P	PP/BU	240	126[g]	ND	—
Toyopearl SP-650C	PM	100	C/I	PP/BU	150	45[h]	ND	—
Toyopearl SP-650M	PM	65	C/I/P	PP/BU	150	50[h]	ND	—
Toyopearl SP-650S	PM	35	I/P	PP/BU	150	50[h]	ND	—
Waters								
Protein-Pak Q HR	PM	8	P	PP	200	—	65	BSA; 20 mM Tris·Cl, pH 8.2
Protein-Pak SP HR	PM	8	P	PP	225	—	40	Cytochrome *c*; 25 mM MES, pH 5.0
Protein-Pak Q HR	PM	15	I/P	PP/BU	200	—	75	BSA; 20 mM Tris·Cl, pH 8.2
Protein-Pak SP HR	PM	15	I/P	PP/BU	225	—	40	Cytochrome *c*; 25 mM MES, pH 5.0
Protein-Pak Q HR	PM	40	B/C/I	BU	200	—	60	BSA; 20 mM Tris·Cl, pH 8.2
Protein-Pak SP HR	PM	40	B/C/I	BU	225	—	20	Cytochrome *c*; 25 mM MES, pH 5.0

continued

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Table 8.2.3 Ion-Exchange Media for Column Purification[a], continued

Medium[b]	Matrix	Avg. bead diameter (μm)	Use	Form available	Ionic capacity (meq/ml)	Available capacity (mg/ml)	Dynamic capacity (mg/ml)	Conditions for determining dynamic capacity
BioSepra								
Q-Hyper D	CP	10	P	PP	250	—	90	5 mg/ml BSA; 50 mM Tris·Cl, pH 8.6; 150 cm/hr; determined at 10% breakthrough
S-Hyper D	CP	10	P	PP	110	—	105	5 mg/ml lysozyme; 50 mM acetate, pH 4.5; 150 cm/hr; determined at 10% breakthrough
Q-Hyper D	CP	20	I/P	PP/BU	250	—	90	5 mg/ml BSA; 50 mM Tris·Cl, pH 8.6; 150 cm/hr; determined at 10% breakthrough
S-Hyper D	CP	20	I/P	PP/BU	100	—	105	5 mg/ml lysozyme; 50 mM acetate, pH 4.5; 150 cm/hr; determined at 10% breakthrough
Q-Hyper D	CP	35	I/P	PP/BU	250	—	40	5 mg/ml BSA; 50 mM Tris·Cl, pH 8.6; 150 cm/hr; determined at 10% breakthrough
S-Hyper D	CP	35	I/P	PP/BU	100	—	100	5 mg/ml lysozyme; 50 mM acetate, pH 4.5; 150 cm/hr; determined at 10% breakthrough
Q-Hyper D	CP	60	C/I	BU	200	—	100	5 mg/ml BSA; 50 mM Tris·Cl, pH 8.6; 150 cm/hr; determined at 10% breakthrough
S-Hyper D	CP	60	C/I	BU	100	—	100	5 mg/ml lysozyme; 50 mM acetate, pH 4.5; 150 cm/hr; determined at 10% breakthrough

[a]Abbreviations: B, batch; BSA, bovine serum albumin; BU, bulk; C, capture; CP, composite polymer, I, intermediate purification; i.d., inner diameter; MES, 2-[*N*-morpholino]ethanesulfonic acid; ND, not determined; P, polishing; PM, polymethacrylate; PP, prepacked; PS/DVB, polystyrene/divinylbenzene.

[b]For addresses and telephone numbers of suppliers, see *SUPPLIERS APPENDIX*.

[c] Prepacked column suitable for use with syringe. For medium specification, see Q Sepharose High Performance.

[d] Prepacked column suitable for use with syringe. For medium specification, see SP Sepharose High Performance.

[e] Prepacked column suitable for use with syringe. For medium specification, see Macro-Prep high Q.

[f] Prepacked column suitable for use with syringe. For medium specification, see Macro-Prep high S.

[g]Conditions for determining available capacity: 5 mg/ml BSA; 50 mM Tris·Cl, pH 8.7.

[h]Conditions for determining available capacity: 5 mg/ml lysozyme; 20 mM phosphate, pH 6.0.

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The number of charged groups on a specified amount of matrix that can participate in ion exchange is termed **ionic** or **total** capacity. However, ion-exchange media are porous and the ion-exchange groups are usually distributed throughout the bead. The amount of protein that will bind is dependent on the ability of the sample components to diffuse into the bead, and this ability is related to the molecular weight and shape of the components and the pore structure of the matrix (see *UNIT 8.3*). In addition, large molecules such as proteins and nucleic acids can bind to multiple ion-exchange groups (or sterically hinder other molecules from binding) depending on the shape and surface-charge distribution of the protein, as well as the spatial orientation of the exchange ligands on the matrix. These factors are taken into account in the available and dynamic capacity of a matrix or column.

Available capacity is defined as the amount of a specific protein that will bind to an ion-exchange medium at a defined pH, salt concentration, and sample concentration in a batch purification process. When medium is packed in a column, the amount of a specific protein that will bind is dependent on many of these factors, as well as on the column dimensions and flow rate. The capacity of a column operating under defined conditions is termed the **dynamic capacity**. The dynamic capacity is typically less than the available capacity for the same sample under the same operating conditions.

Ion-exchange media in which the charge state of the ligand is sensitive to the operating pH are termed weak ion exchangers. The usable pH range of weak ion exchangers is narrower than that of strong ion-exchange media, in which the charge on the ligand used for exchange is constant (i.e., insensitive to pH). Ion-exchange media with DEAE (diethylaminoethyl) or CM (carboxymethyl) groups as the ligand for exchange are considered weak ion exchangers. Because the ionic capacity of weak ion-exchange media is variable, strong ion-exchange media are recommended for pilot experiments (see Support Protocol 1).

When selecting ion-exchange media for batch or column purifications, the amount of medium should be estimated from the manufacturer-supplied capacity data for a protein similar in size to the target protein to be purified. Be aware of what type of capacity (i.e., ionic, available, or dynamic) is being reported and the experimental conditions under which the reported capacity was determined (see Table 8.2.3). A general recommendation is to begin pilot experiments at ~10% of the published capacity data, then determine the capacity for your system.

Preparing Solutions

All aqueous solutions for use in ion-exchange chromatography should be prepared with distilled or deionized water (Milli-Q purified or equivalent). Solutions and samples for column chromatography should be filtered, degassed, and equilibrated to the appropriate temperature prior to use. Special attention must be given to any additives to be included in the buffer to be sure that they will not bind to the column (see preceding discussion on Selecting a Buffer System). If the chosen additives increase the viscosity of the mobile phase, the flow rate should be decreased.

Chromatography Systems

Column techniques typically result in higher resolution than batch techniques, and should be used during the intermediate purification and polishing stages (*UNIT 8.1*) of a purification procedure. However, to achieve high resolution, an appropriate chromatography system is required. A chromatography system may be described simply by the functional operations required to perform a chromatographic separation. The minimum functions required (Fig. 8.2.2) include a buffer delivery system capable of gradient formation, a mechanism for introducing sample into the system, the column itself (properly packed with the appro-

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Table 8.2.4 Scale-Up Calculations for Ion-Exchange Column Chromatography

Scale	1×	10×	100×
Sample			
Sample concentration (mg/ml)	1	1	1
Sample load (mg)	10	100	1000
Sample volume (ml)	10	100	1000
Column			
Column diameter (cm)	0.5	1.6	5
Cross-sectional area (cm^2)	0.2	2	20
Bed height (cm)	5	5	5
Bed volume (ml)	1	10	100
Flow rate			
Volumetric flow (ml/min)[a]	2	20	200
Linear velocity (cm/hr)	300	300	300
Gradient			
Gradient volume (ml)	20	200	2000

[a]Linear flow (cm/hr) multipled by the cross-sectional area of the column (cm^2) equals the volumetric flow rate (ml/hr); cm/hr × cm^2 = cm^3/hr = ml/hr.

priate ion exchanger), an in-line UV monitor suitable for use at 280 nm, a chart recorder, and a fraction collector to preserve the separation. Additional unit operations may include system controllers for automated operation as well as data-acquisition systems. The equipment required to perform each of these unit operations is ultimately determined by the chromatography medium and column selected. Specifically, the operating pressure and flow characteristics of the packed column will be the most important factors for selecting equipment to fit the column, or for selecting columns to fit existing equipment.

It is highly recommended that prepacked columns be used and that the manufacturer be consulted prior to purchase as to equipment requirements for proper operation. This will save time and money and increase the probability of success. If columns are to be packed in the laboratory, the manufacturer's packing instructions for the medium should be followed and column efficiency and function should be tested with representative standards prior to use with the actual sample. There is no single packing methodology that is optimal for all types of media. Table 8.2.3 lists a variety of ion-exchange media available in prepacked columns and/or in bulk for packing in the laboratory. Several manufacturers offer inexpensive disposable ion-exchange columns, suitable for many applications, that can be operated using only a syringe.

Scale-Up Conditions

Ion-exchange separations are typically developed and optimized on a small scale before the entire sample is committed. It is thus important to choose an ion exchanger that will allow simple and convenient scale-up, so that methods established on a small column can be applied more or less directly to a larger one.

Scaling up in ion-exchange chromatography can be approached in two different ways—either the same ion-exchange medium can be used in a larger-diameter column with the same bed height, or a different medium having the same charged group immobilized on a matrix with a higher throughput can be substituted for the medium used in the small-scale separation.

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The former approach, using the same medium throughout, is the simplest. Scaling up a separation with the same medium and bed height used in the small-scale optimization is achieved by employing simple scale factors to adapt the flow, gradient volume, and sample load to the increased volume of a larger-diameter column. The scale factor for the required increase in flow rate is the ratio of the cross-sectional area of the larger column to that of the smaller column (i.e., the factor by which the total flow of solution through the column must be increased so that the same linear velocity used in the small-scale experiment is maintained). The increase in gradient volume and sample loading are directly proportional to the increase in column volume. Table 8.2.4 shows an example of changes that must be made to various parameters for a 10- and 100-fold increase in scale.

BASIC PROTOCOL 1

BATCH ADSORPTION AND STEP-GRADIENT ELUTION WITH INCREASING SALT CONCENTRATION

This protocol describes adsorption of a protein to an ion-exchange medium by direct mixing of the protein-containing sample and the medium (in this case, an anion-exchange gel). This batch purification is in contrast to column purification (see Basic Protocol 2), in which the gel is packed in a column and the sample is passed through it. Batch adsorption is particularly useful where large volumes of sample are involved, which is usually the case in the capture stage of protein purification (*UNIT 8.1*). The equipment used here is sufficient for sample volumes up to 1.5 liter. Larger samples may be divided and processed with parallel apparatuses, or processed sequentially on a single apparatus. Different-sized funnels and side-arm flasks may be used when appropriate.

Once the starting conditions have been determined (see Support Protocol 1), the range of pH and salt concentration where binding and elution occur is well defined. Conditions for batch adsorption and elution used in this procedure are based on the results, presented in Figure 8.2.3, of a test tube pilot experiment performed as described in Support Protocol 1. Buffer volumes and equipment were chosen on the basis of a sample volume of 1 liter containing 50 to 1000 mg of total protein.

Elution of sample components adsorbed to an ion-exchange medium can be achieved either by increasing the salt concentration of the mobile phase, as in this protocol, or by changing its pH (see Alternate Protocol). In batch techniques, discontinuous (step) gradients are used for elution. Column chromatography (see Basic Protocol 2) allows the use of either step or continuous gradients. Formation of continuous gradients requires special equipment (see Basic Protocol 2 and Support Protocol 3).

The number of incremental steps to include in a step gradient must be judged empirically based on the resolution achieved. Ideally (as in the procedure described here), three steps should be used in which all weakly bound materials are desorbed and eluted in the first gradient step using wash buffer, the target protein is eluted in the second gradient step using elution buffer, and all strongly adsorbed materials are eluted in the third gradient step using regeneration buffer. A minimum increment of 50 mM NaCl is recommended for experiments using step-gradient elution with increasing salt concentration. Steps in the gradient may be added, omitted, or modified as determined empirically.

Materials

QAE Sephadex A-25 (Amersham Pharmacia Biotech) or equivalent anion-exchange gel

Binding buffer: 20 mM Tris·Cl, pH 7.5 (or other buffer as determined empirically; see Support Protocol 1)

Protein sample to be purified

Wash buffer: 20 mM Tris·Cl (pH 7.5)/100 mM NaCl (or other buffer/salt solution as determined empirically; see Support Protocol 1)

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Elution buffer: 20 mM Tris·Cl (pH 7.5)/350 mM NaCl (or other buffer/salt solution as determined empirically; see Support Protocol 1)
Regeneration buffer: 20 mM Tris·Cl (pH 7.5)/2 M NaCl (also see Support Protocol 5)

Boiling water bath (optional)
500-ml sintered-glass filter funnel, medium porosity
Three 2000-ml side-arm flasks
Conductivity meter

Swell and equilibrate ion-exchange gel

1. Add 10 g QAE Sephadex A-25 to 1 liter binding buffer and swell 2 days at room temperature or 2 hr in a boiling water bath.

   The volume of swollen gel will be ~70 ml.

2. Mount 500-ml sintered-glass filter funnel on 2000-ml side-arm flask and apply suction.

   Centrifugation (~5000 × g, 1 min) may be used instead of filtration to collect the gel, and may be preferred for processing small sample volumes. It is also possible to simply allow the gel to settle under gravity, then decant or aspirate the supernatant; this may be preferable for very large-scale operations.

3. Gently swirl swollen gel to resuspend. Pour gel slurry into funnel as fast as the fluid level in funnel permits, allowing buffer to collect in flask. Continue until all gel has been collected. Release suction after all buffer has been removed.

   IMPORTANT NOTE: *Never use a magnetic stir-bar to resuspend chromatography medium. This can damage beads by grinding them against the bottom of the vessel, resulting in fine particles that can slow the filtration process.*

4. Add 200 ml binding buffer to funnel and resuspend gel using a stirring rod. Allow resuspended gel to stand for 5 min, then apply suction to remove buffer.

5. Repeat step 4 at least three times to ensure equilibration.

   The gel is considered equilibrated when the pH and salt concentration of the eluant are the same as those of the binding buffer. If the procedures here are followed, there is little need to measure pH and salt concentration of the eluant because the gel is swollen and washed in binding buffer and the chance of error is very small. The worst-case scenario is that the target protein will not bind (see Troubleshooting). The repeated washings are more for the removal of fines than for adjustment of pH or salt concentration.

6. Add enough binding buffer (~100 ml) to produce an ~50% (v/v) slurry and allow gel to stand in funnel until sample has been prepared.

Adsorb sample to gel

7. Adjust pH and salt concentration of protein sample to initial optimal values.

   For a pilot experiment to determine optimal initial values for pH and salt concentration, see Support Protocol 1.

   Samples prepared by salt precipitation may require desalting prior to ion exchange (see UNIT 8.3). The sample can be dialyzed against the binding buffer. Also, the salt concentration of a sample can be reduced by adding distilled water until a desired salt concentration is achieved, as measured by a conductivity meter.

8. Combine gel and sample in 2000-ml beaker or wide-mouth flask.

   The ~50% gel slurry is swirled and poured from the funnel; transfer may be aided with a rubber policeman, spoon, stirring rod, or wash bottle containing binding buffer.

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9. Gently mix sample and gel by swirling every 15 min or shake on a platform shaker at sufficient rate to maintain gel in suspension. Allow 1 to 2 hr for binding at room temperature or 3 to 4 hr at 4°C.

   IMPORTANT NOTE: *Excessive shaking may result in foaming and possible denaturation of the target protein.*

10. Collect gel by filtration as in step 3. Save filtrate for assay.

    This filtrate is saved in case the target protein did not bind. All filtrates should be kept until the fraction containing the target protein has been identified. Storage conditions depend on the stability of the target protein; as a general rule they should be refrigerated to minimize microbial growth. Bacteriostatic agents may be added for prolonged storage.

11. Add 100 ml binding buffer to funnel and resuspend gel using a stirring rod. Allow resuspended gel to stand for 5 min, then apply suction to remove buffer. Repeat three or four times, pooling all washings with the filtrate from step 10.

    The gel containing the bound sample may be packed in a column for subsequent elution steps (see Basic Protocol 2).

Elute with step gradient of increasing salt concentration

12. Transfer the sintered-glass funnel to the top of a clean 2000-ml side-arm flask.

13. Add an equal volume of wash buffer to gel in funnel and resuspend using a stirring rod. Allow resuspended gel to stand for 5 min, then apply suction to remove buffer. Save filtrate.

    The wash buffer is the first step of the gradient. The filtrate will contain sample components that were weakly adsorbed to the gel (see annotation to step 10).

14. Measure absorbance of filtrate at 280 nm using wash buffer as blank.

15. Repeat steps 13 and 14 until the last filtrate shows no significant absorbance at 280 nm. Pool and save the filtrates.

16. Transfer the sintered-glass funnel to the top of a clean 2000-ml side-arm flask.

17. Add an equal volume of elution buffer to gel in funnel and resuspend using a stirring rod. Allow resuspended gel to stand 5 min, then apply suction to remove buffer. Save filtrate.

    The elution buffer is the second step of the gradient. The filtrate will contain the sample components that were more strongly adsorbed to the gel and should include the target protein.

18. Measure the absorbance of the filtrate at 280 nm using elution buffer as a blank.

19. Repeat steps 17 and 18 until the last filtrate shows no significant absorbance at 280 nm. Pool and save the filtrates for assay and subsequent purification of the target protein.

    If gel is to be reused, continue with remaining steps. Otherwise, discard the gel.

Regenerate ion-exchange medium for reuse

20. Transfer the sintered-glass funnel to the top of a clean 2000-ml side-arm flask.

21. Add an equal volume of regeneration buffer to gel in funnel and resuspend using a stirring rod. Allow resuspended gel to stand for 5 min, then apply suction to remove buffer. Repeat five times and discard filtrates.

    The regeneration buffer removes strongly bound materials from the gel. See Support Protocol 5 for additional considerations in regenerating ion-exchange media.

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22. Repeat step 11 for a total of five washes to reequilibrate the gel with binding buffer.

23. Check the pH and conductivity of the last filtrate to ensure that the gel is properly equilibrated.

See Support Protocol 5 for information on storing ion-exchange media.

pH-BASED STEP-GRADIENT ELUTION

ALTERNATE PROTOCOL

The net charge of a protein is pH dependent. Therefore, altering the pH of the mobile phase to make it closer to the pI of a protein can change its net charge, causing it to desorb and elute from an ion exchanger (see Figure 8.2.1). Continuous pH gradients are very difficult to produce at constant salt concentration because charge characteristics of sample components, buffering ions, and ion-exchange media depend on the pH of the system. pH-based step gradients are much easier to produce and more reproducible. For anion-exchange media, elution occurs when the pH is decreased. For cation exchange, the pH is raised for elution.

The first basic protocol can be modified for pH-gradient elution by selecting a buffer at a pH suitable for elution, based on results obtained in a pilot experiment (see Support Protocol 1). This elution buffer is then used in steps 17 to 19 of the first basic protocol. Incubation times and buffer volumes should be increased by 20% when using pH elution.

When scouting methods for ion exchange, it is best to start with changes in salt concentration (see Basic Protocol 1) as this technique is simpler and more reproducible. Elution with pH change is only recommended when the ion-exchange behavior of the sample is well known and the resolution required cannot be attained with a change in salt concentration. pH elution may also be appropriate in cases where it provides a superior ionic/pH environment for loading in the next chromatographic step. A combination of increasing salt concentration and pH change may also be used for elution.

COLUMN CHROMATOGRAPHY WITH LINEAR GRADIENT ELUTION

BASIC PROTOCOL 2

Column chromatography is generally preferred to batch chromatography (see Basic Protocol 1), especially where high resolution is required, although it accommodates smaller sample volumes. Column chromatography requires specialized equipment to achieve high resolution, including a column containing the medium and a fluid-delivery system appropriate for the pressure and flow rate at which the column is to be operated. It is the technique preferred for the intermediate purification and polishing stages of protein purification (*UNIT 8.1*). Also in contrast to the first basic protocol, this technique uses a linear gradient for elution. Although more difficult to produce than step gradients (see Basic Protocol 1), linear gradients lead to better resolution (see Support Protocol 3).

The example presented here uses a RESOURCE Q anion-exchange column from Amersham Pharmacia Biotech, which may be used with either FPLC (fast protein liquid chromatography) or HPLC (high-performance liquid chromatography) systems. Optimal conditions for other columns should be obtained from the manufacturer. Procedures mentioned in the protocol steps should be carried out according to the manufacturer's instructions for the particular chromatography system being used.

NOTE: All buffers, media, and other system components should be filtered, degassed, and equilibrated to the same temperature before use.

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Materials

Liquid chromatography system (FPLC or HPLC)
Elution buffer: binding buffer (see Basic Protocol 1 and Support Protocol 1) containing 1 M NaCl
Binding buffer (see Basic Protocol 1 and Support Protocol 1)
RESOURCE Q chromatography column (1-ml packed bed volume; Amersham Pharmacia Biotech)
Protein sample to be purified

Conductivity meter
0.22-µm filter

Prepare chromatography system

1. Set up the liquid chromatography system according to manufacturer's instructions, without the column in-line.
2. Test system performance by running a blank gradient ranging from 0% to 100% elution buffer in 20 ml at a constant flow rate of 5 ml/min.

   See Figure 8.2.2 for a diagram of a typical column chromatography system. The gradient is formed by placing binding and elution buffer in the appropriate buffer reservoirs (see Support Protocol 3 for detailed discussion of continuous gradient formation).

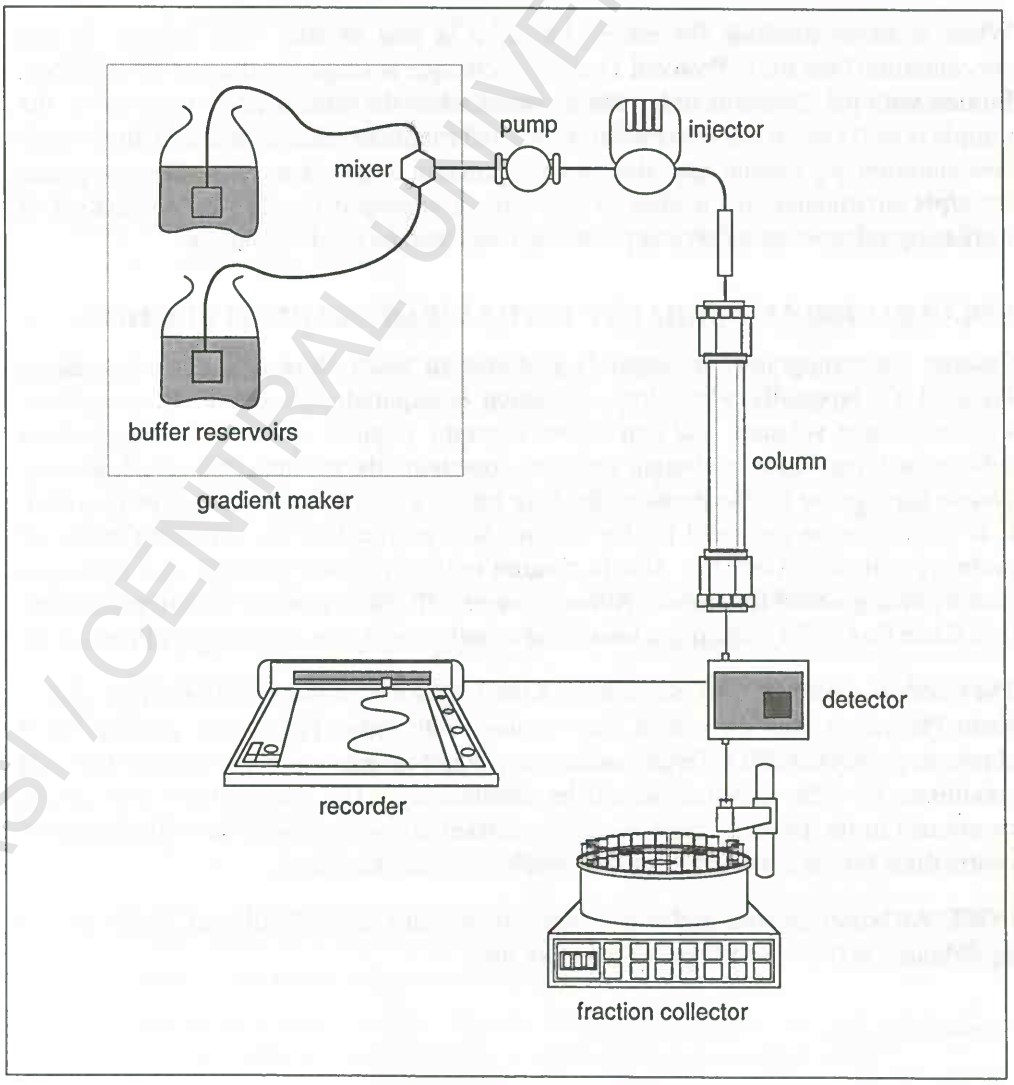

Figure 8.2.2 Liquid column chromatography system with gradient maker.

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Stability of the column at extremes of pH must be considered when choosing binding and elution buffers. The working stability of the RESOURCE Q column is pH 2 to 12, and the stability for cleaning is pH 1 to 14.

The flow rate for the test should be appropriate for the column to be connected. For the RESOURCE Q column, the recommended flow rate is 1 to 10 ml/min and the maximum operating pressure is 4 bar.

3. Test for system leaks and ascertain monitor stability according to system documentation. Test accuracy of gradient composition using conductivity meter.

Adding 0.1% (v/v) acetone to the elution buffer used in step 2 will allow observation of gradient composition at 280 nm using the UV monitor. Do not include acetone in the elution buffer used for the chromatographic run.

Prepare column

4. Purge system with binding buffer to remove any air, then install column in system.

New columns may require precycling prior to use; consult product documentation. Used columns may require cleaning or removal of storage solution prior to use (see Support Protocol 4).

5. Wash column with 5 V_c (5 ml for RESOURCE Q column) of elution buffer at a flow rate of 5 ml/min and check for leakage.

V_c refers to the packed bed volume of a column and is calculated according to the equation $V_c = \pi r^2 \times L$, where r is the radius of the column and L is the bed height (i.e., the height of the packed medium in the column). As the RESOURCE Q column used here has a packed bed volume of 1 ml, the column is washed with 5 ml of elution buffer. Consult product literature to find V_c for other columns.

6. Equilibrate column with 5 to 10 V_c of binding buffer at 5 ml/min. Collect one fraction at the end of the equilibration stage and measure pH and conductivity to ensure the pH and salt concentration of the fraction are the same as that of binding buffer.

If the pH and salt concentration of the fraction are not the same as that of the binding buffer, continue passing binding buffer through the column until equilibration is complete.

Purify protein sample and regenerate column

7. Adjust pH and salt concentration of sample to binding conditions determined in Support Protocol 1. Filter sample prior to injection using a 0.22-μm filter to remove particulates that may clog system or column.

For initial experiments, a sample containing 25 mg total protein is appropriate.

For columns packed with beads of average diameter ≥90 μm, a 1-μm filter may be used; for columns packed with beads of average diameter 34 to 90 μm, a 0.45-μm filter may be used. The 0.22-μm filter is appropriate for columns packed with beads of average diameter <34 μm (e.g., the RESOURCE Q). Alternatively, the sample may be centrifuged 5 min at 20,000 × g to remove particulates.

8. Inject sample into column by opening sample injection valve. Begin fraction collection.

Fractions of 1-ml volume are generally sufficient.

The flow rate during sample injection should be reduced for concentrated samples. It may be increased for dilute samples.

9. After all sample is injected, wash column with 3 to 5 V_c of binding buffer at 5 ml/min to remove unbound or weakly bound materials.

The monitor signal should be allowed to approach the baseline before continuing.

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10. Elute with a linear gradient of 0% to 100% elution buffer in 20 V_c (20 ml).

    Sample-injection valves should be closed to reduce the system dead volume before beginning gradient.

11. Regenerate column by washing with 5 V_c of elution buffer.

12. Reequilibrate column by washing with 5 to 10 V_c of binding buffer.

13. Assay fractions for target protein. Pool fractions containing target protein for further processing.

SUPPORT PROTOCOL 1

TEST TUBE PILOT EXPERIMENT TO DETERMINE STARTING CONDITIONS FOR ION-EXCHANGE CHROMATOGRAPHY

Before beginning an ion-exchange separation (Basic Protocol 1 or 2), the pH and salt concentration required for binding the target protein and the change in pH or salt concentration required for elution must be determined empirically. Once the initial binding conditions have been determined, the available protein capacity (for batch chromatography) is determined to predict the amount of medium that must be used for a given amount of sample. Dynamic capacity (for column chromatography) may be determined as in Support Protocol 2. This protocol is most appropriate when the sample is not strictly limited—e.g., when milligram quantities of a recombinant protein are available. If sample is scarce, a column method should be substituted.

As illustrated in Figure 8.2.3, aliquots of the protein-containing sample to be purified are mixed in test tubes with small quantities of ion-exchange gel that have been equilibrated with a series of buffers of different pH. The optimal binding pH is chosen on the basis of disappearance of target protein in the supernatant at a certain point in the series, then the process is repeated with another series of test tubes containing gel equilibrated with a series of salt solutions of different concentrations. The optimal salt concentrations for binding and elution are chosen, respectively, on the basis of disappearance, then reappearance at another salt concentration, of protein in the supernatant. Finally, aliquots containing increasing protein concentrations are mixed with the gel in a buffer under the optimal binding pH and salt concentrations previously determined. The available capacity of the gel is then determined on the basis of the point where unbound protein appears in the supernatant.

The stability of the target protein at extremes of pH must be considered—i.e., the pH range chosen for the purification should be compatible with retention of structure and activity. In general, this can be evaluated with a crude preparation by incubating aliquots at a series of pH values, then performing the structural or functional assay at the optimal pH value.

NOTE: The method presented here is for anion exchange with increasing salt concentration for elution. Differences for cation exchange are noted where appropriate. Select cation-exchange buffers from Table 8.2.1.

Materials

20 mM piperazine, pH 5.0, 5.5, and 6.0 (prepare from 100 mM stock)
20 mM 1,3-bis[tris{hydroxymethyl}methylamino]propane (bis-Tris propane), pH 6.5 and 7.0 (prepare from 100 mM stock)
20 mM Tris·Cl, pH 7.5, 8.0, and 8.5 (*APPENDIX 2E*; prepare from 100 mM stock)
Q Sepharose Fast Flow (50% slurry in 20% ethanol; Amersham Pharmacia Biotech) or equivalent anion exchange resin in appropriate buffer
Protein sample to be purified, containing known quantity of target and total protein

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4 M NaCl

15-ml test tubes
Centrifuge with rotor accommodating 15-ml test tubes (optional)

Determine optimal binding pH

1. Set up a series of test tubes, numbered 1 to 8, containing different buffers as follows:

   tube 1: 5 ml 20 mM piperazine, pH 5.0
   tube 2: 5 ml 20 mM piperazine, pH 5.5
   tube 3: 5 ml 20 mM piperazine, pH 6.0
   tube 4: 5 ml 20 mM bis-Tris propane, pH, 6.5
   tube 5: 5 ml 20 mM bis-Tris propane, pH 7.0
   tube 6: 5 ml 20 mM Tris·Cl, pH 7.5
   tube 7: 5 ml 20 mM Tris·Cl, pH 8.0
   tube 8: 5 ml 20 mM Tris·Cl, pH 8.5.

2. Shake bottle of Q Sepharose Fast Flow to resuspend gel. Pour 25 ml of gel slurry into a graduated cylinder, then allow gel to settle. Adjust volume of liquid above gel to equal the volume of settled gel (i.e., prepare 50% slurry), then resuspend with a glass stirring rod.

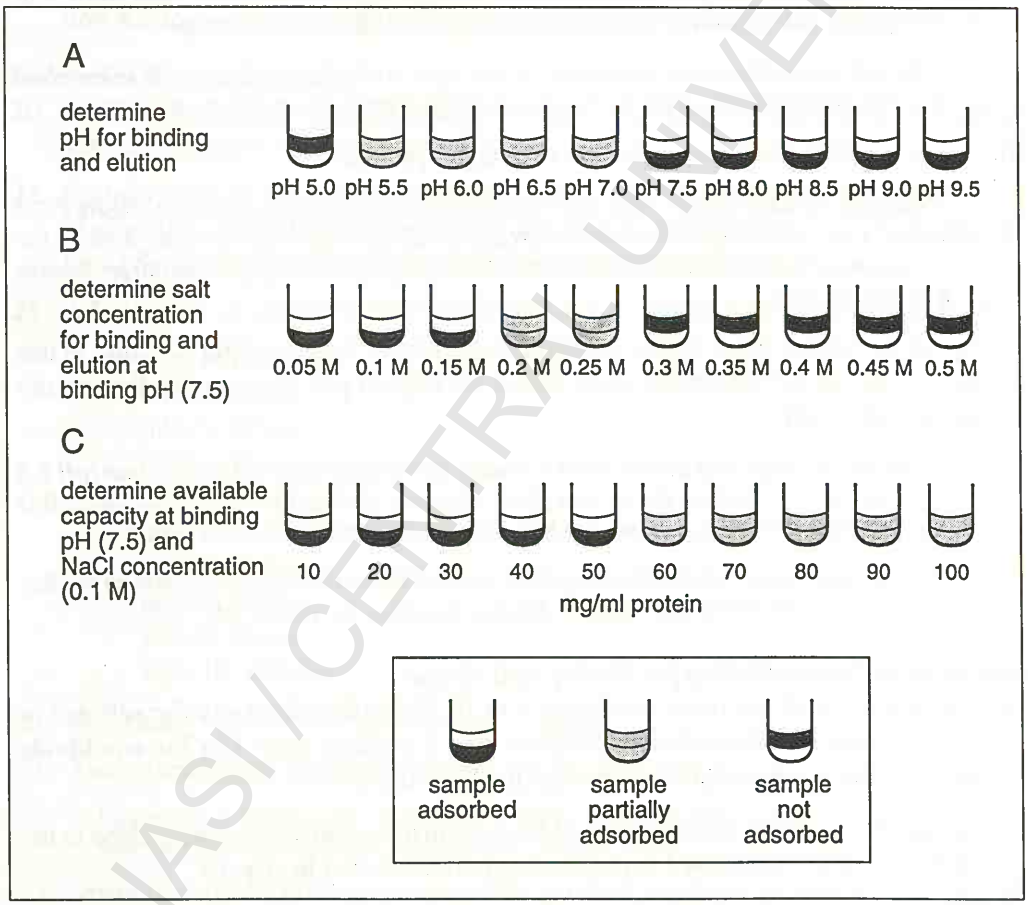

Figure 8.2.3 Results of a typical test tube pilot experiment for selecting initial conditions for anion-exchange chromatography. Series of tubes with protein and ion-exchange medium at (**A**) varying pH (to select binding and elution pH); (**B**) constant pH, varying NaCl concentration (to select binding and elution salt concentrations); (**C**) constant pH and NaCl concentration, varying protein concentration (to determine available capacity). The binding pH here will be ≥7.5; the elution pH will be ≤5.0; the binding salt concentration will be ≥0.15 M; the elution salt concentration will be >0.3 M; and the available capacity will be >50 mg/ml.

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3. Add 2 ml of 50% slurry of Q Sepharose Fast Flow to each tube and mix.

   SP Sepharose Fast Flow (Amersham Pharmacia Biotech) or equivalent may be used for cation exchange.

4. Allow gel to settle to bottom of tube or centrifuge 1 min at ~5000 × *g*, room temperature.

5. Decant supernatant from each tube and replace with 5 ml of the same buffer added in step 1. Gently shake or vortex to resuspend gel, then let tubes stand 2 min.

6. Repeat steps 4 and 5 three times to equilibrate gels in each tube.

7. Decant supernatants and resuspend each equilibrated gel in 1 ml of the same buffer added in step 1.

8. Set up eight identical aliquots (≥1 ml) of the protein to be purified in tubes numbered 1 to 8, each containing 0.1 to 1 mg total protein. Adjust the pH of each to obtain eight different pH values corresponding to those of tubes 1 to 8 in step 1.

   Quantity of protein to be used depends on availability of sample and extinction coefficient of target protein. pH may be adjusted by titrating with acid or base using pH paper or by diluting 1:1 with buffer of desired pH.

9. Add the corresponding pH-adjusted protein aliquot to each of the 8 tubes from step 7. Mix gently for 10 min by periodic swirling, then allow gel to settle.

   If the salt concentration of the sample is too high, no binding will occur. It is therefore recommended that the sample be desalted into the appropriate buffer as in UNIT 8.3.

10. Assay supernatant from each tube for the target protein.

    Measuring the absorbance of the supernatant at 280 nm is usually sufficient. Figure 8.2.3 shows a set of possible results. A decrease or absence of target protein activity (or absorbance) in the supernatant indicates binding. Specific assays for the target protein may also be used.

11. Select the lowest pH at which the highest quantity of target protein is bound to use as the binding pH for further work. Select the highest pH where no binding occurs as the elution pH.

    For anion exchange, it is best to select a binding pH no more than 0.5 to 1 pH unit (pH 7.5 to 8.0 in Fig. 8.2.3) above the highest pH at which no binding occurs. If too high a pH is chosen, elution becomes difficult and high salt concentrations may be required.

    For cation exchange, select a binding pH 0.5 to 1 unit below the lowest pH where no binding occurs. Select the lowest pH where no binding occurs as the elution pH.

Determine salt concentration for binding and elution

12. Set up a series of test tubes, numbered 1 to 10. Using the binding buffer selected in step 10 in place of the different buffers in step 1, perform steps 1 to 7 to equilibrate the Q Sepharose Fast Flow with buffer in all ten tubes.

13. To each tube, add the same amount of the protein to be purified that was added to the tubes in step 8, equilibrated in the binding buffer selected in step 10.

14. Mix contents of tubes gently for 10 min, then allow gel to settle.

15. Decant supernatants from each tube and discard. Wash gel two times with 5 ml of the binding buffer, allowing the gel to settle and decanting the supernatants after each addition of buffer.

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16. Add 2 ml of the binding buffer to each tube, then sequentially add water and 4 M NaCl to each tube as follows:

    tube 1: 1.90 ml H_2O and 0.10 ml 4 M NaCl (0.10 M NaCl final)
    tube 2: 1.80 ml H_2O and 0.20 ml 4 M NaCl (0.20 M NaCl final)
    tube 3: 1.70 ml H_2O and 0.30 ml 4 M NaCl (0.30 M NaCl final)
    tube 4: 1.60 ml H_2O and 0.40 ml 4 M NaCl (0.40 M NaCl final)
    tube 5: 1.50 ml H_2O and 0.50 ml 4 M NaCl (0.50 M NaCl final)
    tube 6: 1.40 ml H_2O and 0.60 ml 4 M NaCl (0.60 M NaCl final)
    tube 7: 1.30 ml H_2O and 0.70 ml 4 M NaCl (0.70 M NaCl final)
    tube 8: 1.20 ml H_2O and 0.80 ml 4 M NaCl (0.80 M NaCl final)
    tube 9: 1.10 ml H_2O and 0.90 ml 4 M NaCl (0.90 M NaCl final)
    tube 10: 1.00 ml H_2O and 1.00 ml 4 M NaCl (1.00 M NaCl final).

17. Mix contents of tubes gently for 10 min, then allow gel to settle.

18. Assay supernatant from each tube for the target protein.

    Figure 8.2.3 shows a set of possible results. Presence of target protein in the supernatant indicates elution of the target protein.

19. Select the highest salt concentration at which no elution occurs as the maximum salt concentration allowable for binding target protein and for washing away unbound sample components. Select a salt concentration at least 0.05 M above the concentration where no protein binds to the gel as the salt concentration for elution.

Determine available capacity

20. Prepare a buffer/salt solution with the binding pH selected in step 10 and the binding salt concentration selected in step 19.

21. Set up a series of test tubes numbered 1 to 10. Using the buffer/salt solution prepared in step 20 in place of the different buffers in step 1, perform steps 1 to 7 to equilibrate the Q Sepharose Fast Flow with buffer/salt solution in all ten tubes.

22. Add aliquots of sample to each tube to obtain the following amount of target protein:

    tube 1: 10 mg
    tube 2: 20 mg
    tube 3: 30 mg
    tube 4: 40 mg
    tube 5: 50 mg
    tube 6: 60 mg
    tube 7: 70 mg
    tube 8: 80 mg
    tube 9: 90 mg
    tube 10: 100 mg.

23. Mix gently for 10 min, then allow gel to settle.

24. Assay supernatant from each tube for the target protein.

    Figure 8.2.3 shows a set of possible results. Presence of target protein in supernatant indicates that the available capacity has been exceeded.

25. Select the highest protein concentration at which the target protein does not appear in the supernatant as the available capacity.

    Calculate the amount of ion-exchange medium needed for batch purification based on 50% of the available capacity (defined in UNIT 8.1). If a column system is used in pilot experiments, however, it is best to determine the dynamic capacity of the medium (see Support Protocol 2). However, 20% of the available capacity is a safe and reasonable starting point for pilot experiments using column chromatography.

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MEASUREMENT OF DYNAMIC (COLUMN) CAPACITY AND BREAKTHROUGH CAPACITY OF ION-EXCHANGE COLUMNS

For chromatographic separations that will be performed on a routine basis as well as separations that will be scaled up, it is necessary first to optimize conditions with respect to resolution and to determine the capacity of the medium with respect to the target protein (see Support Protocol 1). Knowledge of the capacity allows optimal use of the gel medium in terms of cost and yield, and also allows working limits to be set on the sample load to ensure robustness of a particular purification step. After capacity has been determined, the separation can be optimized with respect to time. There are two capacity terms that commonly appear in the literature (in addition to available capacity; also see Support Protocol 1 and *UNIT 8.1*): dynamic capacity and breakthrough capacity.

The dynamic (or column) capacity is the capacity of an ion-exchange column under defined conditions of flow rate, pH, salt concentration and sample concentration. The breakthrough capacity (Q_B) for a system is obtained by calculating the amount of protein that has been absorbed by the column when the sample is first detected in the effluent, or when the recorder signal reaches some arbitrarily defined percent of full-scale deflection. Breakthrough capacity values at 50% full-scale deflection (Q_{B50}) are commonly reported in the literature. However, it is generally recommended to use only 10% to 20% of the published Q_B as the practical capacity of the column to ensure high recovery of the target protein.

Additional Materials (also see Basic Protocol 2)

Ion-exchange gel of unknown capacity, and column
Elution buffer capable of eluting target protein in single step of salt concentration or pH (e.g., 2 M NaCl; see Support Protocol 1)

1. Pack a defined amount of the ion-exchange gel into the column.

   See UNIT 8.3 for guidelines on column packing. Normally, a quantity of gel sufficient to give a packed bed volume of ~1 ml is sufficient. In the case of a prepacked column, the amount of gel is predetermined. The packed bed volume (V_c) is calculated according to the equation $V_c = \pi r^2 \times L$, where r is the radius of the column and L is the bed height (i.e., the height of the packed medium in the column).

2. Prepare chromatography system and column (see Basic Protocol 2, steps 1 to 6) using the optimal binding and elution conditions determined in Support Protocol 1.

   Do not exceed 75% of the flow rate used for packing. The effect of flow rate on capacity should be determined in a true optimization.

3. Prepare protein solution and inject into column (see Basic Protocol 2, steps 7 and 8). Apply solution continually until the recorder shows >50% full-scale deflection, then stop.

   The absorbance of the binding buffer corresponds to 0% and the absorbance of the sample corresponds to 100% full-scale deflection.

4. Wash column with binding buffer until 0% full-scale deflection is approached.

5. Elute proteins with a buffer providing a single-step increase in salt concentration or pH. Continue collecting eluant until recorder shows ≤2% full-scale deflection, then pool fractions.

   A typical chromatogram for this procedure is presented in Figure 8.2.4.

6. Assay pooled fractions from step 5 for target protein. Calculate maximum amount of protein (A) that can be bound to the column according to the equation $A = C_p \times V$,

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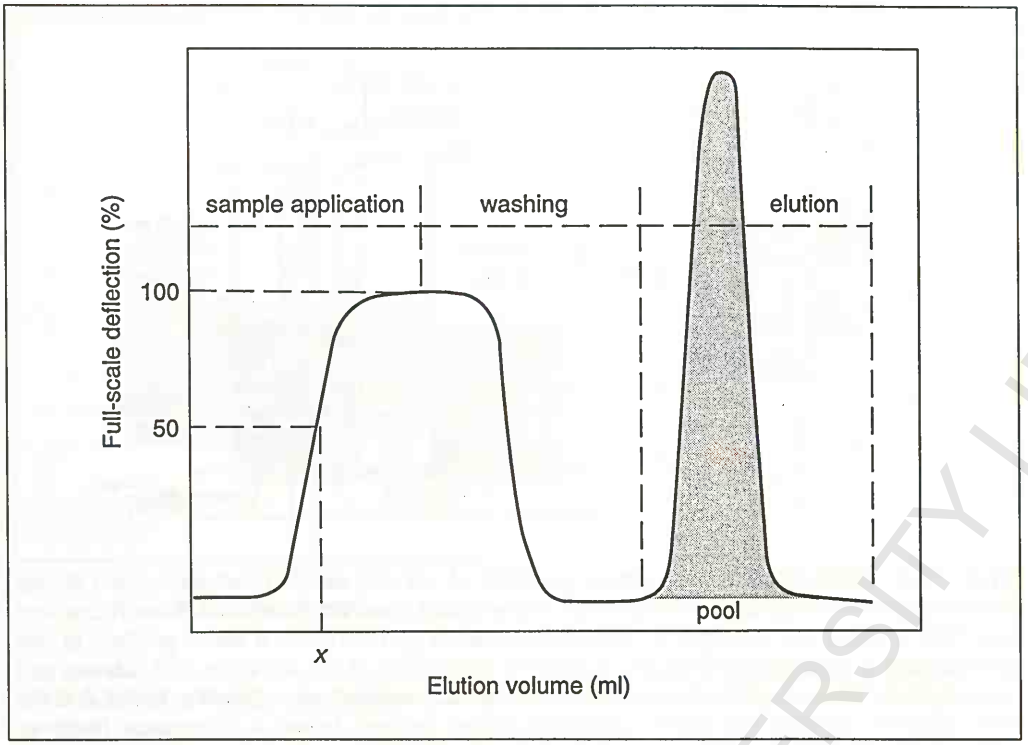

Figure 8.2.4 Typical chromatogram for determining the capacity of an ion-exchange column. The volume of eluant (x) at 50% full-scale deflection is used to calculate the breakthrough capacity (Q_{B50}).

where C_p is the protein concentration of the pooled fractions (mg/ml) and V is the volume of the pooled fractions (ml).

7. Calculate the column (dynamic) capacity (mg/ml) using the equation dynamic capacity = A/V_c.
8. Calculate breakthrough capacity (Q_{B50}) using the equation $Q_{B50} = (C_s \times x)/V_c$, where C_s is the concentration of protein in the original sample and x is the volume of sample that has been applied to the column at the point where 50% full-scale deflection has been attained.

If Q_{B50} greatly exceeds the dynamic capacity, then the target material may still be adsorbed on the column, and the elution conditions should be reexamined.

See Figure 8.2.4 for graphical depiction of these variables.

GRADIENT-FORMATION TECHNIQUES

SUPPORT PROTOCOL 3

Simple Gradient Mixers

Gradient mixers of the type shown in Figure 8.2.5 can generate linear pH or salt gradients in gravity-based or one-pump chromatography systems. Similarly designed gradient mixers used for casting electrophoresis gels may also be used for chromatography. The reservoir closest to the column is filled with the binding buffer and the second reservoir is filled with an equal volume of the elution buffer. The gradient begins when the valve between the two reservoirs is opened. Continuous mixing is required in the reservoir closest to the column to ensure gradient linearity and reproducibility.

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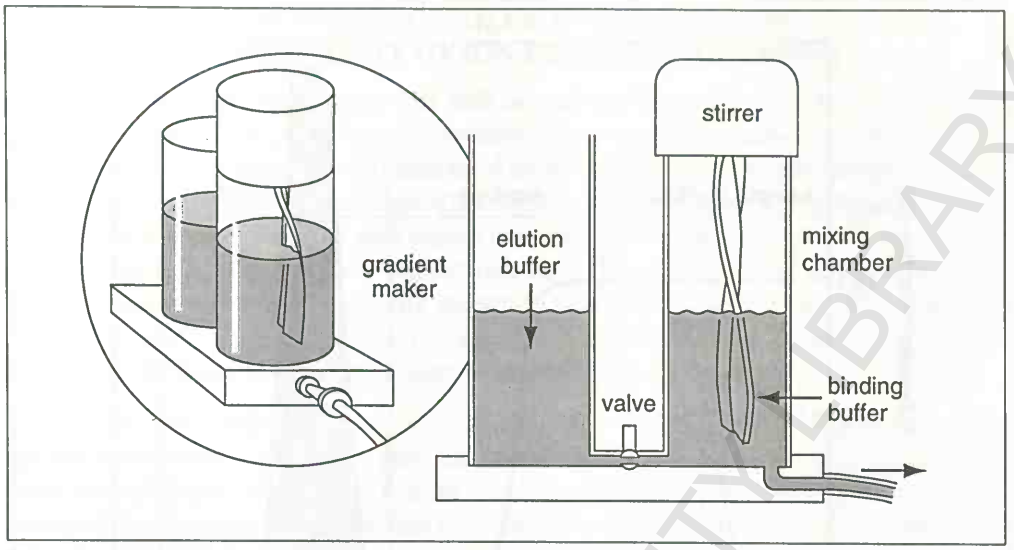

Figure 8.2.5 Gradient mixer for forming gradients of pH and salt concentration used during ion-exchange chromatography. The apparatus shown is an Amersham Pharmacia Biotech Gradient Mixer GM-1, which can be used for preparing gradients up to 500 ml. A linear gradient of salt concentration is produced by filling the reservoirs with buffers at the same pH and different salt concentration. The mixing chamber contains the lower-salt-concentration (binding) buffer, and the other chamber contains the higher-salt-concentration (elution) buffer. A continuous linear or nonlinear pH gradient may be produced by filling the reservoirs with buffers at the same salt concentration and different pH. A gradient of increasing pH is used for cation-exchange separations, and a gradient of decreasing pH is used for anion-exchange separations. The buffer chambers are joined by a channel controlled by a valve, and outflow from the mixing chamber is controlled by another valve.

Gradient Mixing with Multichannel Peristaltic Pumps

Peristaltic pumps that can accommodate three or more pumping channels simultaneously (Fig. 8.2.6) can be used to form linear or complex gradients (see Critical Parameters). Linear gradients are easily formed by using the same size tubing for all channels, pumping with one channel from the elution buffer reservoir to the binding buffer reservoir, and pumping with the other two channels from the binding buffer reservoir to the column. Continuous mixing is required in the binding buffer reservoir closest to the column to ensure gradient linearity and reproducibility.

Switch-Valve-Based Gradient Mixing

Switch-valve-based gradient formation is useful for forming step, linear, and complex gradients (see Critical Parameters). Gradients are formed by proportioning variable amounts of binding buffer and elution buffer through a single three-way valve using a system controller. When programmed to deliver 10% elution buffer, the controller will open the valve port for binding buffer 90% of the time and the port for elution buffer 10% of the time. As higher switching rates are required for lower flow rates to ensure gradient accuracy, high-quality system controllers will vary the rate at which the valve switches (i.e., the number of switching events per unit time) as a function of flow rate. A dynamic mixer should be included in the flow path just after the proportioning valve to ensure gradient accuracy. Switch-valve-based gradients are generally not very accurate from 1% to 10% elution buffer (90% to 99% binding buffer) or from 1% to 10% binding buffer (90% to 99% elution buffer). Therefore, the ionic strength of the binding and elution buffers may be adjusted to allow a gradient of the desired composition to be formed by mixing the two buffers in the range of 20% to 80% elution buffer (80% to 20% binding buffer).

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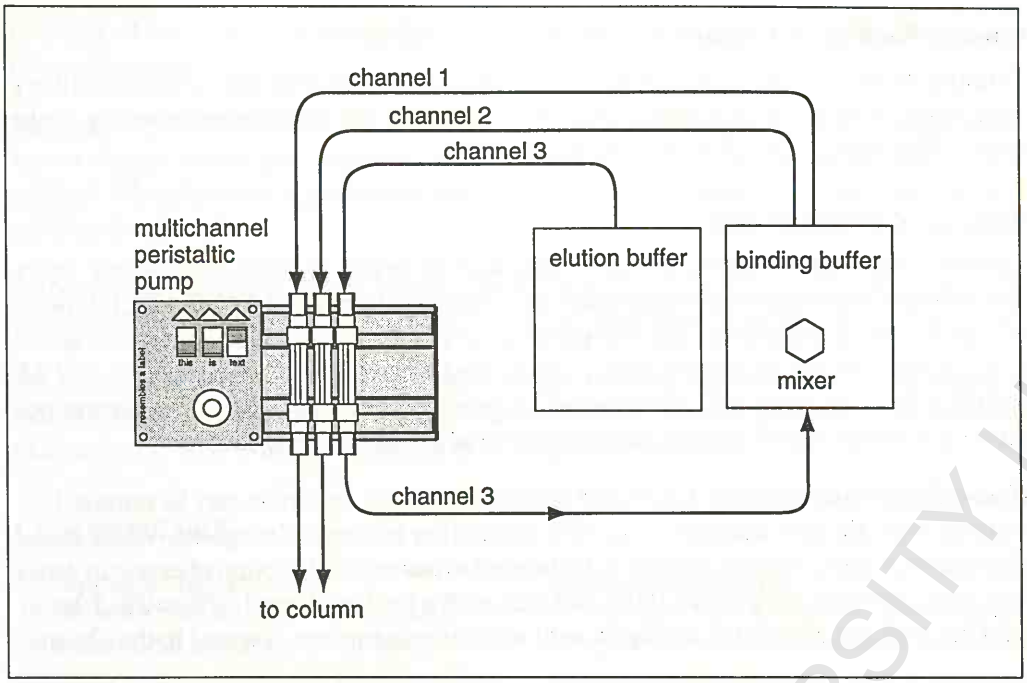

Figure 8.2.6 Peristaltic pump accommodating three pumping channels for continuous gradient formation.

Gradient Formation by Autoblending

Some chromatography systems allow gradient formation over a range of both pH and salt concentration. Four solvents—a high-pH buffer, a low-pH buffer, a concentrated salt solution, and water—are blended via four two-way valves. A system controller proportions the high- and low-pH buffers to achieve the desired pH gradient while simultaneously proportioning the concentrated salt solution and water to achieve the desired salt-concentration gradient. Gradients formed in this way are usually difficult to reproduce but may be useful for scouting initial binding and elution conditions.

Gradient Formation with Twin Pumps

Use of two positive-displacement pumps, as in HPLC and FPLC systems, provides the highest degree of accuracy, precision, and reproducibility in gradient formation. One pump delivers binding buffer while the other delivers elution buffer. A system controller allows gradient formation simply by controlling the flow rate of each pump. A dynamic mixer should be included in the flow path, after the pumps and before the column, to ensure gradient accuracy.

CLEANING AND REGENERATION OF ION-EXCHANGE MEDIA

SUPPORT PROTOCOL 4

Proper maintenance of chromatography media is as much an art as protein purification itself. Knowing what kind of contaminating material from the sample is bound to the column helps in selecting a regime for maintaining good column hygiene. It is best to practice preventative maintenance by regenerating the medium after every run, using a high-salt buffer or large change in pH, and periodically cleaning the column and checking the system flow characteristics. If any increase in column back-pressure is noted, the column should be cleaned as soon as possible. Once enough contaminant has bound to a column to reduce or block the flow, it is probably too late to save the medium without investing considerable time and effort.

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Routine Washing of Columns and Media

Columns or bulk medium should be washed after every run with salt solution until an ionic strength of ≥2 M is reached. This should remove any substances bound by ionic forces. This is recommended after every run.

Removal of Contaminants

Alkali-soluble contaminants. Contaminants such as lipids, proteins, and nucleic acids can usually be removed by washing with 2 to 3 column volumes of 0.1 M NaOH, followed by 2 to 3 column volumes of Milli-Q-purified (or equivalent) distilled water and then 2 to 3 column volumes of binding buffer. Many media can tolerate brief exposure to 1 M NaOH, if harsher conditions are required to remove contaminants; however, manufacturer's guidelines should be consulted regarding pH stability of the medium.

Hydrophobic contaminants. Lipids and other hydrophobic materials may be removed by washing with alcohol solutions (e.g., 70% ethanol) or nonionic detergents. When using alcohols or organic solvents to remove hydrophobic materials, it is often effective to wash first with a gradient from 0% to 100% and then with a gradient from 100% to 0%. Repeat washing with the alternating gradients until no contaminants are detected in the eluant.

Metallic contaminants. Contamination with metals may cause blue or gray discoloration at the top of an ion-exchange column. These metals can originate from impurities in buffer salts or water, or they can leach from metallic system components in contact with buffer solutions. Metal contaminants may usually be removed by treating the column with several column volumes of 10 mM HCl (i.e., pH 2) saturated with EDTA. Manufacturer's guidelines should be checked for pH stability of medium and column before exposure to mild or strong acid solutions.

Precipitated materials. Precipitated materials that have accumulated in a column are very difficult to remove. Unless the precipitated material can be physically removed by removing the gel at the top of the column, the material will have to be resolubilized. Detergents, urea, and guanidine·Cl can be introduced to help dissolve contaminants. The viscosity of concentrated solutions used for cleaning may require very low flow rates. The column may be equilibrated and incubated in 6 M urea, then washed with distilled water and buffer. Degradative enzymes (e.g., proteases and lipases) may also be introduced in an attempt to degrade precipitates on the column; however, enzymatic cleaning can be quite expensive. It may be useful to reverse the direction of flow through the column (i.e., from bottom to top) when attempting to remove particulate material from the top of the bed.

SUPPORT PROTOCOL 5

STORAGE OF ION-EXCHANGE MEDIA

Bacterial and microbial growth can seriously interfere with the chromatographic properties of any chromatography medium and endanger the sample as well. Prior to storage, all media should be cleaned and sanitized. During prolonged experiments or storage (i.e., >24 hr at room temperature or 48 hr at 4°C), a bacteriostatic or antimicrobial agent should be added to the ion exchanger. Antimicrobials chosen to be added for storage should not bind to the ion exchanger and should be easily removed when the gel is to be reused. Azide is anionic and will bind to anion-exchange media.

Treatment with 1 M NaOH for 1 hr will sanitize most media by lowering the bacterial count 100-fold, and will help solubilize dead cells (Pharmacia Biotech, 1997). Storage of sanitized media in 0.1 M NaOH will not leave any toxic materials on the column—a significant consideration in pharmaceutical production. Manufacturer's guidelines should

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be checked for stability of the medium and column in NaOH. Silica-based media will not tolerate high pH.

Exposure to 70% ethanol for 3 to 4 hr can be used to sanitize most media. Most ion-exchange media and columns can be stored for prolonged periods of time in 20% ethanol. Manufacturer's guidelines should be checked for stability of the medium and column in ethanol.

Effective antimicrobial agents appropriate for use with anion-exchange media include either 0.001% phenyl mercuric salts or 0.002% chlorhexidine, in weakly alkaline solution.

An effective antimicrobial agent for use with cation-exchange media is 0.005% merthiolate in weakly acidic solution. An agent that is effective for use with either anion or cation exchangers is 0.05% trichlorobutanol in weakly acidic solution.

COMMENTARY

Background Information

The first ion exchangers were synthetic resins designed for applications such as demineralization, water treatment, and recovery of ions from waste. Early ion-exchange resins were tightly cross-linked hydrophobic polymers, highly substituted with ionic groups, and had very high capacity for small ions. The high degree of cross-linking provided mechanical strength, but the limited porosity restricted use of these media with large molecules. In addition, the high charge density resulted in very strong binding, and the hydrophobic matrix tended to adsorb and denature labile biological materials. The first ion exchangers designed for use with biomolecules were developed by Peterson and Sober (1956). These cellulose-matrix ion exchangers were very hydrophilic and had little tendency to denature proteins, but had low capacities and poor flow characteristics. Modern ion-exchange media have very high capacity, often >100 mg/ml. Modern particle technology has significantly increased the mechanical strength and flow characteristics of ion-exchange media so that separations that once required hours or days may now be completed in a few minutes.

Ion-exchange can easily be coupled to other methods of protein separation in a multistep purification. Samples purified by ion exchange can typically be applied to a hydrophobic-interaction chromatography (HIC) medium simply by adding NaCl (or other salts) to a concentration sufficient for binding (*UNIT 8.4*). Samples to be further purified by chromatofocusing or an additional round of ion exchange will require desalting (*UNIT 8.3*). Samples may usually be applied directly to gel-filtration columns (*UNIT 8.3*) with no intervening treatment.

Critical Parameters

The most critical aspects of any ion-exchange experiment are selection of the appropriate ion-exchange medium and of the initial conditions for binding and elution of the target protein or contaminants. Once these parameters are selected, conditions can be optimized with respect to the three primary parameters of any chromatographic separation—i.e., resolution, capacity, and speed. These three parameters are mutually exclusive. Resolution will typically decrease when the sample load is increased and when the flow rate is increased (to increase speed). It is generally recommended that resolution be optimized first, then capacity, and finally speed.

Optimization of resolution

This parameter is most easily optimized by controlling the selectivity (*UNIT 8.3*) of the medium through manipulation of binding and elution conditions. Construction of chromatographic titration curves followed by optimization of the shape of the elution gradient is a common approach to achieving maximum resolution.

Chromatographic titration curves can be constructed by performing a series of anion- and cation-exchange experiments at different pH values as in Figure 8.2.7. For each of these experiments, the elution salt concentrations (in mM NaCl) for sample components A, B, and C are plotted on the *y* axis and the operating pH is plotted on the *x* axis. It is then simple to observe the pH ranges within which optimal resolution between the various sample components is achieved. The different sample components are usually detected by UV absorption, but may also be viewed by other means (e.g., fluorescence or bioactivity). Data for chroma-

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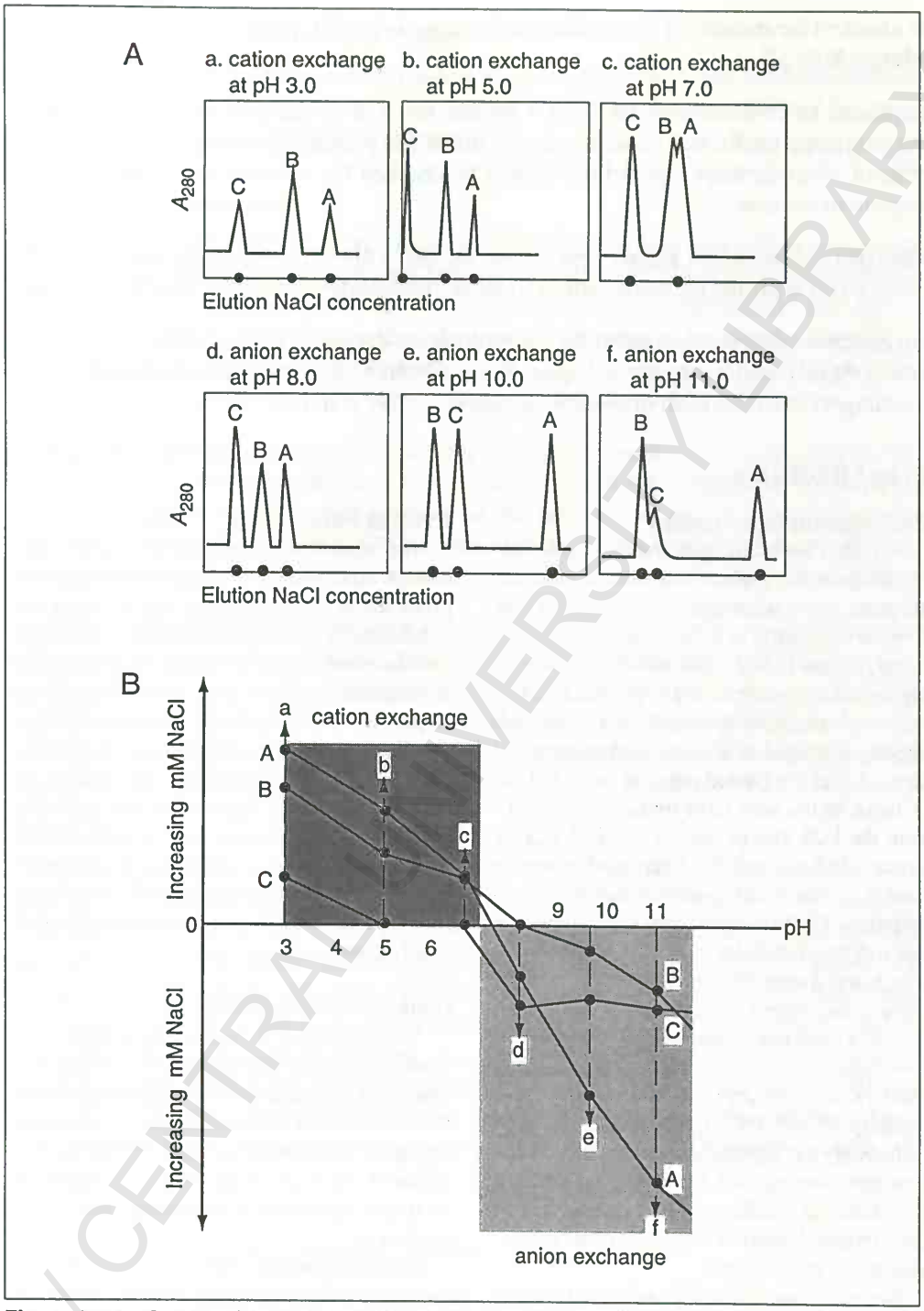

Figure 8.2.7 Construction of a chromatographic titration curve. (**A**) Six chromatographic separations (a to f) of a sample containing proteins A, B, and C are carried out at six different operating pHs using a linear gradient of increasing salt concentration. (**B**) The elution ionic strengths for components A, B, and C (*y* axis) are plotted against the operating pH (*x* axis) for experiments a to f. Note that the salt concentration in mM NaCl (*y* axis) is ascending in both directions. The optimal resolution with respect to component A occurs below pH 5 with cation exchange and above pH 9 with anion exchange.

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tographic titration curves can often be generated by fast screening with low-volume gradients (e.g., 5 to 10 V_c) and low sample mass (e.g., <1 mg).

After optimization of resolution with respect to pH, the shape of the elution gradient can be optimized. A gradient can be conceptually divided into segments, and each segment may have a different gradient shape. Gradient shapes include linear, concave, convex, and step. Complex gradients are constructed by using different gradient shapes in consecutive segments. The slope (e.g., mM NaCl per minute) of a linear segment is constant, that of a concave segment continually increases, that of a convex gradient continually decreases, and that of a step gradient approaches infinity. Resolution between sample components in any segment can usually be increased by decreasing the gradient slope in that segment. However, decreasing the gradient slope will increase the peak volume during elution, the gradient volume, and the time of the separation. Step gradients can provide the most concentrated fractions, and are desirable when minimizing sample volume is of primary importance. Use of concave gradients in the initial part of a segment and convex gradients in the later part of a segment can improve resolution. The capabilities of available equipment must be considered in the construction of complex gradients. Use of simple gradients that do not stress the performance limits of the system will result in robust methods that are insensitive to small changes in sample or buffer composition and gradient shape, thereby increasing probability of success.

To construct a complete gradient profile, it is first necessary to identify the gradient range where desorption of the target protein occurs, as well as the gradient slope that provides optimal resolution. A single-step elution is then done at a salt concentration just below the range of where the target protein elutes, to remove weakly bound material. The target protein is then desorbed and eluted. A single-step elution at a high salt concentration will then remove tightly bound sample components and prepare the ion-exchange gel for regeneration. This approach, using step gradients before and after the range of interest, can provide simple, robust methods that are easy to scale up, and will save time and reagents.

Different ion-exchange media offer different selectivities (*UNIT 8.1*). If adequate resolution for a specific stage in a purification cannot be obtained with a specific ion-exchange medium, it may be desirable to select an alternate ion exchanger with a different functional group, or to use a different chromatographic technique at this stage in the separation. Salts other than NaCl may be used in elution, but there are no specific effects from different salts on the separation in ion-exchange chromatography, other than differences in concentration. Karlsson et al. (1998) provides a good review of the effects of nonbuffering ionic species in ion exchange.

Optimization of capacity

When optimum resolution has been achieved, the effect of increasing sample load on resolution should be evaluated to determine the optimal balance of resolution and capacity utilization. The procedure for determining the dynamic (column) capacity is described in Support Protocol 3 and can be performed using the conditions for optimal resolution (as determined above) to elute the target protein. Once the capacity is determined under the optimal elution conditions, the effect of changes in sample load on resolution can be investigated to identify optimal conditions. It is generally recommended that only 10% to 20% of the dynamic capacity be used to ensure high recoveries of the target protein.

Optimization of throughput

Once optimal conditions for resolution and capacity have been determined, the flow rate in each gradient segment may be increased and the effect on resolution and capacity monitored to complete the optimization process.

Troubleshooting

Common problems encountered during ion-exchange experiments, their potential causes, and suggested solutions are outlined in Table 8.2.5.

Anticipated Results

A chromatogram illustrating typical results of an ion-exchange column chromatography experiment (i.e., Basic Protocol 1) is shown in Figure 8.2.8.

The goal of any purification step is the pure target protein obtained at the end of the day. The chromatogram is only a record for comparison and is deceptive at best. Great care should be exercised in interpreting a chromatogram—i.e., the results from assays performed on fractions collected must be used in interpret-

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Table 8.2.5 Troubleshooting Guide for Ion-Exchange Chromatography

Problem	Possible cause	Solution
Protein does not elute in the salt gradient	Buffer pH is incorrect	Use buffer pH closer to pI of the protein
	Salt concentration is too low	Use more concentrated elution buffer
Protein does not elute in pH gradient	Buffer pH is incorrect	Calibrate pH meter and prepare new solutions
Protein elutes in wash phase	Salt concentration of binding buffer is too high	Decrease salt concentration of binding buffer
	Salt concentration of sample is too high or pH is wrong	Perform buffer exchange on sample
	Column is not properly equilibrated	Repeat or prolong equilibration until conductivity is constant
	Ionic detergents or other additives are absorbed to column	Clean column
Resolution is less than expected	Gradient slope is too steep	Use shallower gradient or include plateau in gradient
	Flow rate is too high	Run separation at lower flow rate
	Proteins or lipids have precipitated on columns	Clean and regenerate column
	Sample has not been filtered properly before application to the column	Regenerate column, filter sample, and repeat chromatography
	Proteins in sample have formed aggregates that bind strongly to gel	Use urea or detergents to clean column
	Column is poorly packed	Check packing by running a colored compound and observing the band. Repack column if necessary.
	Too much sample mass has been loaded onto column	Clean and regenerate column. Decrease sample load and repeat run.
	Detector flow cell volume or mixing spaces in or after column are too large	Change flow cell or reduce all post-column volumes
Leading or very rounded peaks observed in chromatogram	Column is overloaded	Decrease sample load and repeat run
	Column is poorly packed	Check packing by running a colored compound and observing the band. Repack column if necessary.
	Column needs regeneration	Clean and regenerate column. If this does not help, replace with new one.
Tailing of peak is observed in the chromatogram	Sample is too viscous	Reduce amount of protein, or remove nucleic acids from sample.
	Precipitation of protein has occurred in column filter and/or at top of the gel bed	Clean column; exchange or clean filter.
	Column is poorly packed	Check packing by running a colored compound and observing band. Repack column if necessary.
Previous elution profile cannot be reproduced	Buffer pH and/or ionic strength are incorrect	Prepare new solutions
	Sample has deteriorated during storage	Prepare fresh sample
	Proteins or lipids have precipitated on column	Clean and regenerate column

continued

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Table 8.2.5 Troubleshooting Guide for Ion-Exchange Chromatography, continued

Problem	Possible cause	Solution
	Sample has not been filtered properly	Regenerate column, filter sample carefully, and repeat step
	Equilibration was incomplete	Repeat or prolong equilibration until conductivity is constant
Recovery of activity is low but recovery of protein is normal	Target protein is not stable in the elution buffer and is therefore inactivated	Change elution conditions
	Enzyme separated from cofactor or other substance needed for activity	Test by pooling all fractions and repeating assay
	Microbial growth has occurred in the column	Clean column and store gel in 20% ethanol or other antimicrobial agent[a]
Amount of protein in the eluted fractions is much less than expected	Protein was degraded by proteinases	Add proteinase inhibitors to buffers
	Protein adsorbed to filter during sample preparation	Use another type of filter or add detergents to buffer
	Microbial growth occurred in the column	Clean column and store gel in 20% ethanol or other antimicrobial agent[a]

[a]Microbial growth rarely occurs in columns during use, but steps should always be taken to prevent infection of packed columns, buffers, and gel suspensions; see Support Protocol 5.

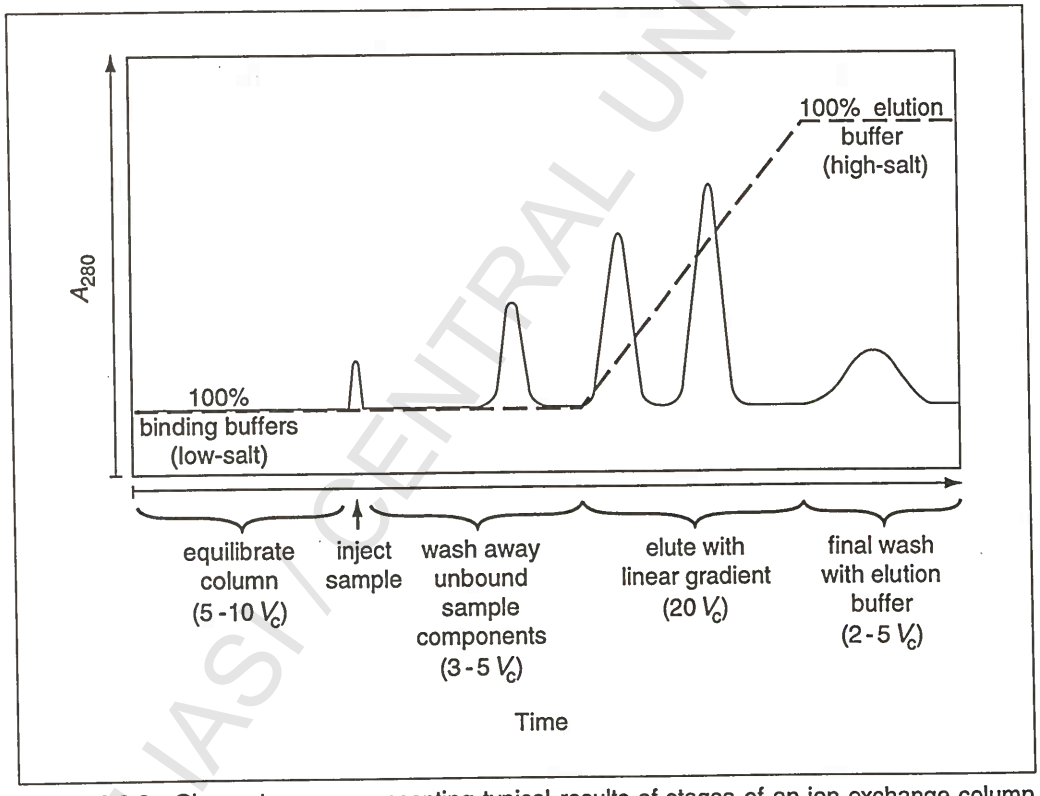

Figure 8.2.8 Chromatogram representing typical results of stages of an ion-exchange column chromatography experiment. A linear gradient of 0% to 100% elution buffer is used as in Basic Protocol 2. The broken line superimposed on the chromatogram represents the composition of the elution gradient (100% binding buffer to equilibrate column and remove unbound sample components, followed by a linear gradient from 100% binding buffer to 100% elution buffer to elute bound proteins and a final wash with 100% elution buffer). V_c represents the packed bed volume of the column.

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ing the chromatogram and inferring the identity of any given chromatographic peak.

Time Considerations

If all necessary equipment is available and functioning properly, it should be possible to develop a chromatographic method in a day. With automated systems (e.g., FPLC and HPLC) and modern ion-exchange media, method development may require just a few hours. However, the most time-consuming aspect in developing a purification step is typically the determination of protein purity in the fractions collected, which will always depend on the specific protein of interest. The key to minimizing the time required for such determinations is to strive for high resolution and a minimum number of fractions.

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Enzymatic Approaches for Obtaining Amino Acid Sequence: On-Target Ladder Sequencing

Peptide sequencing by mass spectrometry (MS) is usually based on detecting mass differences associated with various amino acids in the polymer chain. Post-source decay (PSD) and MS/MS spectra may yield internal peptide sequences. However, determination of the order of the first two, and sometimes the last few, amino acids in the peptide is often problematic without additional experiments. Several enzymatic approaches have proven useful for identifying the N- and C-terminal residues (Patterson et al., 1995; Thiede et al., 1995; Woods et al., 1995). They involve the use of carboxypeptidases and aminopeptidases to produce peptide ladders for rapid analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

The ladder sequence method described in the Basic Protocol may generate sequence information from low- to subpicomole quantities of peptides and can be performed directly on the sample stage, thus minimizing potential sample losses to vials and through sample transfer. It should be pointed out that success is not predictable because of the substrate specificity of the enzymes. However, the experiments are easy to perform and the resulting mass spectra easy to interpret.

BASIC PROTOCOL

Materials

Carboxypeptidase P, Y, and A
50 mM sodium citrate buffer, pH 6.0
Aminopeptidase M
50 mM ammonium carbonate buffer, pH 7.0
~1 pmol/µl peptide substrate in 0.1% (v/v) trifluoroacetic acid (TFA)
Matrix solution (*UNIT 16.3*, Basic Protocol 1)
10% (v/v) formic acid, cold
Matrix-assisted laser desorption/ionization (MALDI) mass spectrometer

1. Prepare C-terminal sequencing enzyme in sequencing buffer immediately before use:

   0.1 to 0.2 µg/µl carboxypeptidase P in 50 mM sodium citrate buffer, pH 6.0
   0.1 to 0.2 µg/µl carboxypeptidase Y in 50 mM sodium citrate buffer, pH 6.0
   0.1 to 0.2 µg/µl carboxypeptidase A in 50 mM sodium citrate buffer, pH 6.0.

   Carboxypeptidases have different preferences for each amino acid, causing the kinetics of enzymatic release of different amino acids to vary considerably. Therefore, it is advisable to use different carboxypeptidases in parallel in a time-monitored fashion to release all amino acids in an interpretable manner.

   When the amount of sample is limited and C-terminal sequence information is needed, start with carboxypeptidase Y (as this enzyme can cleave all C-terminal residues) or use a mixture of all three enzymes.

2. Prepare N-terminal sequencing enzyme in sequencing buffer immediately before use:

   2.0 µg/µl aminopeptidase M in 50 mM ammonium carbonate buffer, pH 7.0

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3. Apply 0.3 µl of ~1 pmol/µl peptide substrate in 0.1% TFA to a clean sample target for each time point and enzyme to be used. Add an equal volume of sequencing enzyme to each.

   At relatively high peptide concentrations (≥1 pmol/µl) the incubation may also be performed in a microcentrifuge tube from which aliquots are subsequently taken and applied to the target at different time points.

   For six time points and four enzymes, a total of twenty-four spots is needed.

4. Incubate sample target for 9, 15, 30, 60, 90, and 600 sec at room temperature.

   Lower concentrations of substrate generally require shorter incubation periods with enzymes to sequentially cleave the same number of residues from both the C- and N-terminal ends. The above time points are suitable for ~1 pmol/µl of peptide substrate.

   It is advisable to perform the digestion time course in a humidified chamber, as spots of the longer incubations may dry up.

5. To end the digestion, add 0.6 µl matrix solution to the digest mixture, mix by pipetting up and down, and allow to air dry.

6. Rinse the matrix/sample preparations with 10 µl cold 10% formic acid (*UNIT 16.3*, Basic Protocol 2, step 4).

7. Insert the sample target into the mass spectrometer and perform mass analysis according to the instrument manufacturer's instructions (*UNIT 16.3*, Basic Protocol 1).

COMMENTARY

Background Information

Enzymatic sequencing is an alternative approach to chemical sequencing or fragmentation induced in the mass spectrometer. Carboxypeptidases offer a simple method by which amino acids can be sequentially cleaved from the C terminus of a protein or a peptide. Carboxypeptidase Y is a particularly attractive enzyme as it nonspecifically cleaves all residues from the C terminus, proline included. Aminopeptidase M is a metalloprotease completely hydrolyzing peptides and proteins with a free α amino group and L amino acids (X-Pro bonds are not cleaved). Enzymatic sequencing was historically performed by analysis of the released amino acids, which was complicated by amino acid contaminants and required relatively large amounts of sample. Using mass spectrometric techniques such as MALDI-MS, however, it is possible to perform direct mass analysis of the peptide fragments resulting from carboxypeptidase digestion. In this ladder sequencing approach, a sequence can be read, in the correct order, by calculating the differences in mass of adjacent peptide peaks. Enzymatic sequencing has been performed mostly in a time-dependent approach (see Basic Protocol), although concentration-dependent carboxypeptidase digestion has been successfully used for enzymatic sequencing as well (Patterson et al., 1995). Both digestion approaches can be conveniently performed on the MALDI sample target, minimizing the amounts of sample and enzyme consumed and losses of sample to vials.

Critical Parameters

Carboxypeptidase digestions often need to be optimized to obtain the maximum sequence information in the shortest amount of time. For example, when the enzyme concentration is too high, sequence gaps may occur for adjacent amino acids that are readily cleavable. Lower concentrations of enzyme may allow for all amino acids to be sequenced, but often require long periods of time (up to days) for total digestion.

Mass spectrometry cannot differentiate between leucine and isoleucine or between lysine and glutamine because they have identical molecular weight. Moreover, the confidence with which amino acid sequences are determined depends on the instrumental mass accuracy. For MALDI mass analysis without delayed extraction, depending on the amino acids, multiple measurements may be needed for reliable assignments. For example, more analyses are required for mass differences of 113 to 115 Da (Ile/Leu, Asn, and Asp) and 128 to 129 Da (Glu/Lys/Glu) than for mass differences around

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163 (Tyr) and 57 (Gly), when excluding all but one assignment as statistically unlikely. See Patterson et al. (1995) for statistical considerations.

Anticipated Results

Using MALDI-MS analysis of on-target carboxypeptidase digestions, peptides of various amino acid composition, size, charge, and polarity have been successfully C-terminally sequenced at low picomole levels (Patterson et al., 1995; Thiede et al., 1995; Woods et al., 1995). Due to the salt content of the enzyme/buffer solution, prominent MNa^+ ions may be detected in addition to MH^+ ions when the carboxypeptidases are used at 0.2 µg/µl.

Time Considerations

The amount of digestion time needed to obtain C-terminal or N-terminal sequence information of a peptide or protein may vary from a few minutes to many hours.

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Key Reference

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This article provides an elaborate description of both time-dependent and concentration-dependent digestion strategies. In addition, it provides a protocol for statistical analyses of the resulting peptide mass ladder.

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PREPARATION AND HANDLING OF PEPTIDES

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Native Chemical Ligation of Polypeptides

The total synthesis and semisynthesis of proteins allows the site-specific incorporation of unnatural amino acids, post-translational modifications, and biophysical/biochemical probes into the target molecule. Among the various chemical and enzymatic approaches available for the synthesis/semisynthesis of proteins, the native chemical ligation technique has proven especially useful and will be the exclusive focus of this unit. Native chemical ligation allows native backbone proteins to be assembled from fully unprotected polypeptide building blocks. To facilitate the ligation reaction, the α-carboxylate group of the N-terminal polypeptide fragment must be mildly activated as an aryl thioester, while the C-terminal polypeptide fragment must contain an amino-terminal cysteine residue. The reaction is normally carried out in aqueous buffers at around neutral pH and can be performed with complete regioselectivity in the presence of all the functionalities commonly found in proteins. Even the presence of additional cysteine sulfhydryl groups in one or both peptide fragments does not affect the reaction regioselectivity. The unit first discusses how to choose the ligation site(s) in the target protein and then outlines how to obtain the necessary polypeptide building blocks using either chemical synthesis or recombinant DNA expression (see Strategic Planning). Next, the synthesis of a protein by native chemical ligation of two polypeptide fragments is described (see Basic Protocol 1). The synthesis of a protein from three polypeptide fragments using a sequential native chemical ligation strategy is also described (see Basic Protocol 2). The support protocols describe how to obtain the necessary polypeptide fragments using either chemical synthesis or recombinant DNA expression.

NOTE: All the operations involving volatile chemicals should be performed in a well ventilated fume hood. See *APPENDIX 2A* for general safety guidelines.

STRATEGIC PLANNING

The first step in synthesizing a protein by native chemical ligation is to choose the ligation site(s), thereby defining the polypeptide fragments to be used in the ligation reactions. The position of the ligation site depends upon several factors: the primary sequence of the protein; the secondary and tertiary structures of the protein (if known); and the type of strategy being used to generate the fragments, i.e., chemical synthesis or recombinant DNA expression. Ideally the target protein should be divided up into synthetically accessible fragments the size of which depends upon whether chemical synthesis or biosynthesis is being used for their generation; optimized solid-phase peptide synthesis (SPPS; also see *UNIT 18.1*) allows polypeptides of up to ~50 residues to be efficiently prepared, whereas recombinant DNA expression permits the preparation of much larger fragments. For example, a hypothetical 100-amino-acid protein could be assembled either from two ~50-residue synthetic peptides or from a 20-residue synthetic fragment and an 80-residue recombinant fragment. Note that in the latter strategy (i.e., semisynthesis) it is important to design the synthesis in such a way that the chemically synthesized fragment encompasses the region of the protein to be engineered. Where possible, naturally occurring X-Cys motifs in the native sequence should be chosen as the ligation sites, where X can be any residue except Asp, Asn, Glu, Gln, and Pro. In the absence of an endogenous Cys at the appropriate position in the primary sequence, it will be necessary to mutate a native residue in order to facilitate ligation. The effect of this Cys mutation on the structure and function of the protein can usually be minimized by following a few simple rules: (1) If the tertiary structure of the protein is known, choose a region remote from the active site of the protein, preferably in a flexible surface loop; (2) choose the mutation to be as conservative as possible, e.g., Ser→Cys or Ala→Cys; (3) try to avoid

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Table 18.4.1 Resins and Amino Acid Derivatives Usually Employed in the Synthesis of N-Terminal Cys and α-Thioester Polypeptides[a]

Polypeptide	N^{α}-protecting group strategy	Side-chain protecting groups	Resin(s)	C-terminal functionality after cleavage
N-terminal Cys	Boc	Arg(Tos), Asn(Xan), Asp/Glu(OcHx), Cys(Meb), Gln(no protection), His(Dnp), Lys(2-Cl-Z), Met(no protection), Ser/Thr(Bzl), Trp(For), Tyr(2-Br-Z)	MBHA PAM	—$CONH_2$ —COOH
	Fmoc	Asn(Trt), Arg(Pmc), Asp/Glu(O*t*Bu), Cys(Trt), Gln(Trt), His(Trt), Lys(Boc), Met(no protection), Ser/Thr/Tyr(*t*Bu), Trp(Boc)	Rink amide Wang	—$CONH_2$ —COOH
α-thioester	Boc	As in the Boc strategy for obtaining N-terminal Cys polypeptides, except for His(Bom) and Trp(no protection or Hoc)	3-mercapto-propionamide -MBHA	—COS-$CH_2CH_2CONH_2$

[a]Abbreviations: Boc, *t*-butyloxycarbonyl; Bom, benzyloxymethyl; 2-Br-Z, 2-bromobenzyloxycarbonyl; *t*Bu, *t*-butyl; 2-Cl-Z, 2-chlorobenzyloxycarbonyl; Dnp, 2-4-dinitrophenyl; Fmoc, 9-fluorenyloxycarbonyl; For, formyl; Meb, *p*-methylbenzyl, O*t*Bu, *t*-butyl ester; OcHx, cyclohexyl ester; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Tos, tosyl (*p*-toluenesulfonyl); Trt, trityl; Hoc, cyclohexyloxycarbonyl; PAM, 4-hydroxymethylphenylacetamidomethyl; Xan, xanthyl.

mutating residues that are conserved across a gene family; and (4) take advantage of any known mutational data on the system since the effect on structure/function of mutating a particular residue may already be known.

Chemical synthesis and recombinant DNA expression each allow the generation of polypeptides containing an amino-terminal cysteine residue (C-terminal fragment) and the generation of polypeptides containing an α-thioester functionality (N-terminal fragment). For polypeptides of ~50 residues or less, chemical synthesis is usually the method of choice since it is relatively quick and efficient and allows the incorporation of unnatural amino acids. Amino-terminal cysteine–containing peptides can be chemically synthesized via established *t*-butyloxycarbonyl (Boc) or 9-fluorenyloxycarbonyl (Fmoc) SPPS protocols using commercially available resins (e.g., from NovaBiochem, Bachem, Peptides International, Applied Biosystems, or Neosystems) and amino acid derivatives (see Table 18.4.1). The synthesis of peptide α-thioesters can currently only be achieved using Boc chemistry, due to the sensitivity of the thioester functionality to the base-deprotection conditions associated with the Fmoc-SPPS strategy. Note, the resin required for the synthesis of peptide α-thioesters is not commercial available, but can be prepared in the laboratory in a single day.

For polypeptide fragments larger than ~50 amino acids in length, a biosynthetic strategy should be employed. It must be noted that this assumes the gene has been cloned. Polypeptides containing amino-terminal Cys residues can be obtained using a mutagenesis/proteolysis strategy. This involves constructing an expression vector in which a DNA sequence encoding the peptide Ile-Glu-Gly-Arg-Cys is inserted between a upstream affinity-purification handle (e.g., GST or His tag) and the appropriate gene fragment of interest (for cloning strategies see *UNIT 6.6*). Following expression and affinity purification (*UNIT 6.6*), the fusion protein is treated with Factor Xa to give the desired recombinant Cys-polypeptide. Polypeptides containing α-thioester functionalities can be obtained by expressing the corresponding polypeptide sequence using the IMPACT expression system (New England Biolabs). This results in the generation of a fusion protein in which the polypeptide of interest is attached through its C-terminus to an intein-CBD, where the intein is an engineered protein-splicing element and CBD (chitin binding domain) is an

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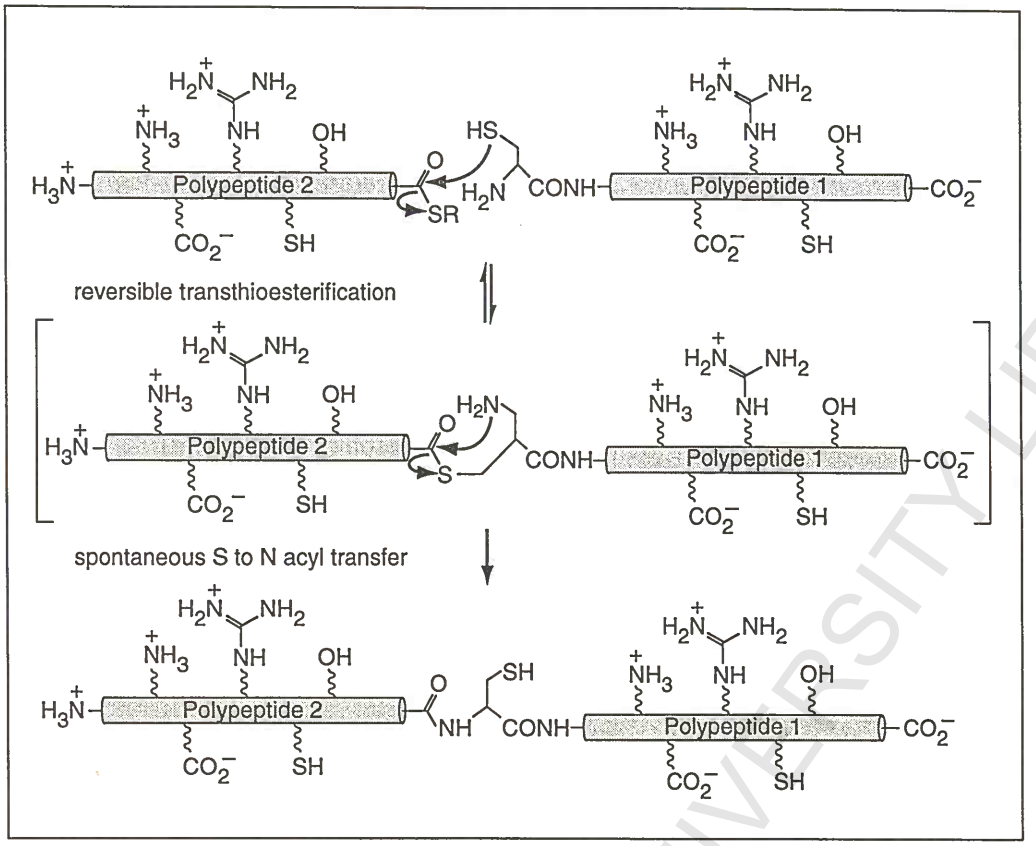

Figure 18.4.1 The mechanism of native chemical ligation. The initial step is a reversible transthioesterification reaction involving the thiol group of the N-terminal Cys-polypeptide (C-terminal fragment) and the α-thioester moiety of the N-terminal polypeptide fragment. This intermediate undergoes a spontaneous rearrangement to form a natural peptide bond at the ligation site.

affinity handle. The desired recombinant α-thioester polypeptide can be obtained by simply treating the immobilized fusion protein with an appropriate thiol; the intein-CBD construct remains attached to the affinity matrix.

NATIVE CHEMICAL LIGATION OF TWO POLYPEPTIDES

BASIC PROTOCOL 1

This protocol describes the chemical ligation of two polypeptide segments to afford a target protein containing both fragments linked through a native peptide bond. The reaction is initiated by dissolving both peptides in aqueous buffer at pH 7.5 in the presence of thiol cofactors. The reaction usually takes between 1 and 3 days to go to completion, at which point the product is purified using reversed-phase high-performance liquid chromatography (RP-HPLC) and subsequently folded. A schematic of the chemistry behind the procedure is presented in Figure 18.4.1.

Materials

6 M guanidine·HCl buffer (see recipe)
Nitrogen source (ultrapure)
Benzyl mercaptan (Aldrich)
Thiophenol (Aldrich)
Polypeptide with N-terminal Cys residue (see Support Protocols 1 and 2)
Polypeptide with α-thioester functionality (see Support Protocols 3 and 4)
1 M NaOH
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 99% pure; Strem Chemicals)

continued

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Buffers for analytical C18 reversed-phase HPLC
Buffer A: H_2O containing 0.1% trifluoroacetic acid (TFA)
Buffer B: 1 part H_2O/9 parts acetonitrile/0.1% TFA
Appropriate final buffer in which target protein will be dissolved (e.g., for long-term storage, activity studies, or structural studies), containing 6 M guanidine·HCl and 5 mM DTT
Appropriate final buffer in which target protein will be dissolved, containing 4 M, 2 M, and 0 M guanidine·HCl (*APPENDIX 3A*) and 1 mM DTT

Dialysis membrane with MWCO substantially smaller than target protein
Centricon filter (Amicon; optional)
Tabletop centrifuge (Sorvall RT-7 or equivalent)

Additional reagents and equipment for analytical *and* semipreparative or preparative C18 reversed-phase HPLC, electrospray ionization (ESI) or matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS; *UNITS 16.1 & 16.2*), dialysis (*UNIT 4.4 & APPENDIX 3B*), and spectrophotometric determination of protein concentration (*UNIT 3.1*)

Prepare the ligation buffer

1. Degas the 6 M guanidine·HCl buffer by purging with ultrapure nitrogen for at least 20 min.

2. Add benzyl mercaptan and thiophenol, each at a concentration of 0.5% (v/v), to the degassed buffer. Mix by vortexing to produce the ligation buffer.

   IMPORTANT NOTE: *This ligation buffer should be prepared just prior to use and should not be stored for long periods of time. Thiols are, in general, toxic and have an unpleasant odor; therefore thiol-containing solutions should be treated with 5% (v/v) commercial bleach in 1:1 (v/v) methanol/water before being disposed of.*

   Since benzyl mercaptan and thiophenol are barely soluble in aqueous buffers, vortexing is required to form a homogeneous thiol suspension in the ligation buffer.

Ligate the N-terminal and C-terminal polypeptide fragments

3. Dissolve equimolar amounts of the polypeptides to be ligated (i.e., one with an N-terminal Cys residue and the other with an α-thioester functionality) in the ligation buffer to a final concentration of at least 0.5 mM with respect to each peptide.

   Reactions can be run in volumes of 50 to 100 µl for analytical purposes and up to 0.5 to 5 ml for preparative purposes.

   Native chemical ligation reactions are usually carried out in the presence of chaotropic agents (guanidium·HCl or urea) in order to maximize the concentration of the two reactants and to increase the availability of reacting moieties by alleviating any steric hindrance that might be associated with folded polypeptides. The use of chemical denaturants, of course, presumes that the final product can be properly folded in vitro. In cases where the fragments being ligated must be kept in a folded state, the ligation reaction can be carried out in the absence of chemical denaturants.

4. Check the pH of the ligation mixture by spotting a 2- to 3-µl aliquot onto a strip of pH paper. If it is above or below 7.0, adjust the pH by titrating in small increments with 1 M HCl or NaOH, respectively. Repeat this process until a pH of ~7.5 is reached.

   Dissolving lyophilized peptides at high concentrations can cause the pH of the ligation mixture to drop; this is particularly so when HPLC-purified materials are being used (i.e., from acidic conditions). It is extremely important to ensure that the pH of the reaction mixture is kept between 7 and 8, since the efficiency of the native chemical ligation is strongly dependent on the pH. As the pH drops below pH 7.0, the reaction slows down, and as it increases above pH 8.0 the chemoselectivity of the process is lost.

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5. Keep the reaction at room temperature with gentle stirring for 24 hr.
6. After 24 hr, take a 5-µl aliquot of the reaction mixture (allowing the rest of the mixture to continue reacting as in step 5) and dilute the aliquot in a polypropylene microcentrifuge tube with 50 µl of 6 M guanidine·HCl buffer in the presence of one crystal of tris-(2-carboxyethyl)phosphine hydrochloride (TCEP, used as a reducing agent). Shake the sample until the TCEP is completely dissolved, wait 10 min, then microcentrifuge 5 min at 10,000 rpm.
7. Analyze 25 µl of the supernatant by analytical C18 reversed-phase HPLC, initially using a linear gradient from 0% buffer B to 73% buffer B over 60 min at a flow rate of 1 ml/min and refining as necessary based on the elution times of the reactants and product. Monitor the run via UV detection at 214 nm (and 280 nm if aromatic residues are present in the target protein). Manually collect every peak (except the solvent peak) into a separate polypropylene microcentrifuge tube and analyze each by ESI-MS (*UNITS 16.1 & 16.2*), which will allow the retention times of the reactants and the product to be determined

   Occasionally the peak corresponding to the ligation product is buried beneath the thiophenol or benzyl mercaptan peaks. This can usually be resolved by optimizing the HPLC gradient or, alternatively, by lyophilizing the reduced sample a few times and then reanalyzing it by HPLC.
8. After 36 hr take another 5-µl aliquot of the reaction mixture and analyze it by HPLC as in step 7. If significant amounts of both reactants still remain and the product peak area is still increasing, then leave the reaction for another 12 hr and repeat the analysis. If, on the contrary, both reactants have been consumed or if the product peak area has not increased appreciably, then terminate the reaction as in step 9.

   Ligation reactions normally take 24 to 36 hr to go to completion, although on rare occasions longer periods (3 to 4 days) or shorter periods (a few hours) may be required.
9. Dilute the crude reaction mixture into 5 to 10 vol of 6 M guanidine·HCl buffer and reduce by adding 15 molar equivalents of TCEP (i.e., based on the quantities of reactants used; 15 times the number of moles of either one of the polypeptides added). Shake the solution until all the TCEP is dissolved and wait for 20 min.
10. Centrifuge the sample 20 min at 3000 rpm in a tabletop centrifuge (Sorvall RT-7 or equivalent), room temperature. Retain the supernatant.

    A white cloudiness often appears in the reaction medium during the course of the reaction. This is due to the slow formation of insoluble benzyl mercaptan and thiophenol disulfides. The reactants and ligation product will generally remain in solution, allowing the unwanted disulfides to be removed by centrifugation.

    The crude ligation mixture can be stored at –80°C for 2 to 3 days, although it is advisable to purify it as soon as possible.

Purify the target protein

11. Purify the crude reaction mixture by semipreparative or preparative reversed-phase HPLC using an optimized gradient system. Analyze each HPLC fraction by mass spectrometry (*UNITS 16.1 & 16.2*) and analytical reversed-phase HPLC in order to identify those fractions containing the target protein. Pool the fractions of highest purity (>95%) and lyophilize.

    Reversed-phase HPLC purification will denature the majority of proteins, meaning that a subsequent refolding step is required. In cases where the ligation reaction has been carried out under more physiological conditions (i.e., without chemical denaturants in the ligation buffer), the final purification step can be performed using, e.g., gel-filtration chromatography (UNIT 8.3) or ion-exchange chromatography (UNIT 8.4) under conditions compatible with protein folding.

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Fold the purified protein

12. Dissolve the lyophilized target protein to a final concentration of ≤100 μM in the appropriate freshly degassed buffer (e.g., for long-term storage, activity studies, or structural studies), containing 6 M guanidine·HCl and 5 mM DTT.

    Although the present protocol involves the stepwise dialysis from 6 M guanidium·HCl, it should be stressed that this approach may not work for all proteins and that a certain amount of optimization will be required for every system. It should also be noted that for disulfide-containing proteins, an additional oxidation step will have to be performed.

    In general, the lower the final concentration of protein the better, since this will tend to minimize aggregation during the refolding step.

13. Dialyze the solution at 4°C (*UNIT 4.4 & APPENDIX 3B*) against the same buffer as in step 12, this time containing 1 mM DTT and decreasing amounts of guanidine·HCl (i.e., first against 4 M guanidine·HCl, then against 2 M guanidine·HCl, then against final storage buffer). In each step perform dialysis for at least 12 hr and change the buffer 2 to 3 times during this period.

    The MWCO of the dialysis membrane should be chosen to be substantially smaller than the purified protein.

14. Remove sample from dialysis bag, centrifuge to remove any precipitate, and quantify the supernatant by UV spectrophotometry at 280 nm (*UNIT 3.1*). If necessary concentrate the protein solution using a Centricon filter (*UNIT 4.4*; Amicon), then divide into aliquots and freeze at –70°C.

BASIC PROTOCOL 2

SEQUENTIAL CHEMICAL LIGATION OF THREE POLYPEPTIDES

If the region of interest in the protein is >50 residues from the N- or C-termini, then its chemical modification will be extremely difficult using a two-fragment ligation strategy, since this would require the chemical synthesis of a peptide >50 residues in length. This problem can be overcome by assembling the target protein from three polypeptide fragments using a sequential native ligation strategy. The basic method for sequential native ligation uses a N-terminal Cys polypeptide (C-terminal fragment), an α-thioester polypeptide (N-terminal fragment), and an N^{α}(methylsulfony)ethyloxycarbonyl-Cys, α-thioester polypeptide (central fragment). The procedure starts by ligating the middle fragment and the C-terminal fragment at pH 7.5 in the presence of thiol cofactors. The N^{α}(methylsulfony)ethyloxycarbonyl (Msc) group is then removed and the resulting polypeptide (central plus C-terminal fragment) is ligated with the N-terminal fragment to give the target protein, which is purified and refolded. The general approach is depicted in Figure 18.4.2.

Materials

Three polypeptide fragments to be ligated:
  Polypeptide with N-terminal Cys residue (see Support Protocols 1 and 2)
  Polypeptide with N^{α}(Msc)-Cys, α-thioester functionality (see Support Protocol 5)
  Polypeptide with α-thioester functionality (see Support Protocols 3 and 4)
1 M HCl

Additional reagents and equipment for native chemical ligation of two polypeptides (see Basic Protocol 1)

1. Prepare the ligation buffer (see Basic Protocol 1, steps 1 and 2). Ligate the central [i.e., N^{α}(Msc)-Cys, α-thioester] and C-terminal (N-terminal Cys) polypeptide fragments [see Basic Protocol 1, steps 3 to 8, but use the N^{α}(Msc)-Cys, α-thioester polypeptide in the reaction mix in place of the α-thioester polypeptide].

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2. When the ligation reaction is complete, remove the N^{α}-Msc protecting group by raising the pH of the crude ligation mixture to 13 with 1 M NaOH solution (see Basic Protocol 1, step 4). After 1 min, lower the pH to 5.0 to 7.0 with 1 M HCl (use ~1.1 to 1.2 times the volume of 1 M NaOH required to raise the pH to 13).

   Usually, for a 1-ml ligation mixture, the pH can be raised by adding 75 µl of 1 M NaOH, then dropped by adding 95 µl of 1 M HCl. It is recommended that the pH be dropped by adding the acid all at once, instead of by titration, for reasons of speed.

   The presence of thiols improves the yield in the deprotection step, since they trap the still reactive methyl ethylenyl sulfone side product.

3. Analyze the crude reaction mixture (see Basic Protocol 1, steps 6 and 7) and examine to see if the Msc deprotection is complete. Purify the polypeptide fragment (see Basic Protocol 1, steps 9 to 11), which will consist of the central fragment ligated to the C-terminal fragment.

4. Starting with the lyophilized (central plus C-terminal) polypeptide fragment prepared in steps 1 to 3 above and the N-terminal polypeptide fragment (containing an α-thioester functionality), perform the ligation reaction to generate the target protein (see Basic Protocol 1, steps 3 to 10).

5. Purify and refold the target protein (see Basic Protocol 1, steps 11 to 15).

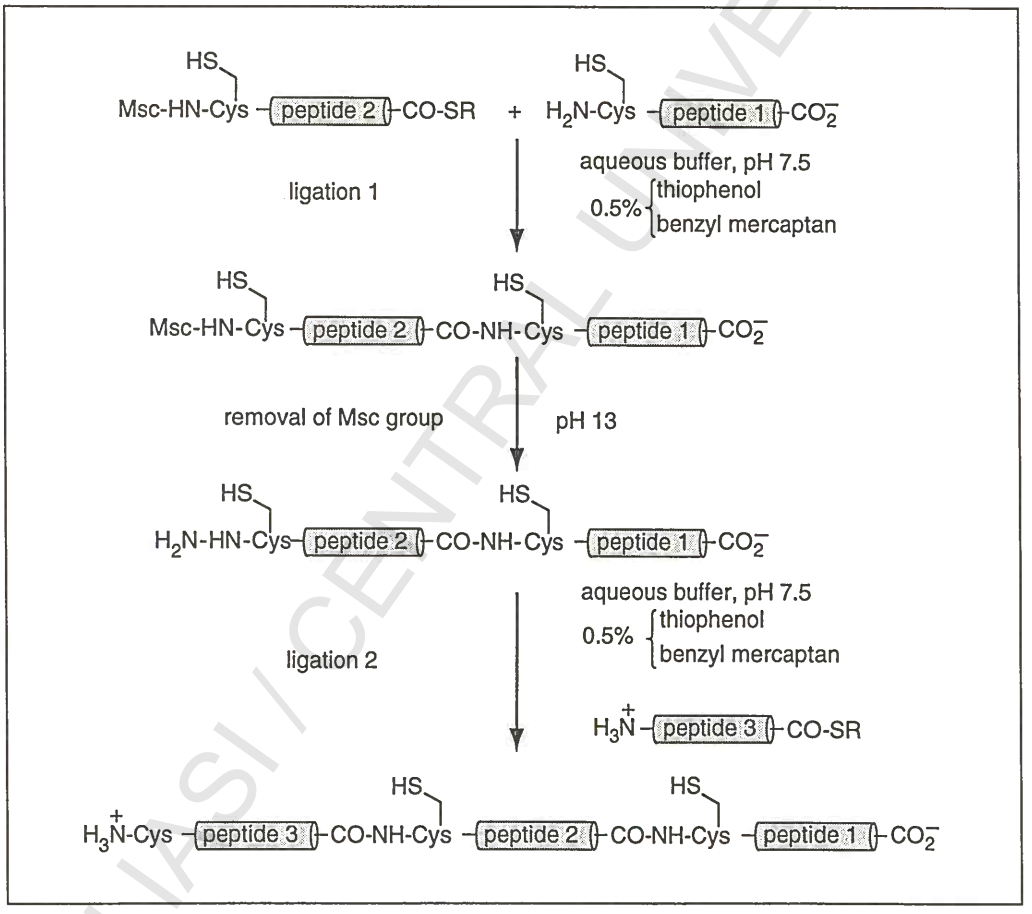

Figure 18.4.2 The principle of sequential native chemical ligation. The key to this approach is the reversible protection of the α-amino group of the central peptide fragment, thereby preventing self-reaction with the α-thioester moiety present in the same molecule. This can be accomplished with the base-labile Msc [N^{α}(methylsulfony)ethyloxycarbonyl] group, which can be easily removed by brief treatment with base after the first ligation step. The newly deprotected fragment is now ready for the next ligation step.

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SUPPORT PROTOCOL 1

CHEMICAL SYNTHESIS OF N-TERMINAL CYS-POLYPEPTIDES

The chemical synthesis of N-terminal Cys-polypeptides can be easily carried out using standard SPPS with Boc or Fmoc N^{α}-protected amino acids and the appropriate resins (see Table 18.4.1 and *UNIT 18.1*). In both cases, the synthesis can be performed on automated solid-phase peptide synthesizers, which are available in the core facilities of many institutions.

NOTE: Extreme care must be taken to avoid exposing N-terminal Cys-polypeptides to even trace amounts of carbonyl-containing compounds (e.g., acetone or formaldehyde). These chemicals react rapidly with the α-amino and thiol groups of the N-terminal Cys to give a very stable thiazolidine derivative which prevents the peptide from participating in subsequent native chemical ligation reactions. For the same reason, the groups benzyloxymethyl (Bom) and *t*-butyloxymethyl (Bum), used to protect the side-chain of His in Boc and Fmoc SPPS, respectively, should be avoided during the synthesis of these polypeptides since they release formaldehyde during the cleavage/deprotection step.

SUPPORT PROTOCOL 2

BIOSYNTHESIS OF N-TERMINAL CYS POLYPEPTIDES

Biosynthesis represents a complementary approach to chemical synthesis (see Support Protocol 1) for obtaining N-terminal-Cys polypeptides, and is especially useful when the target polypeptide fragment is somewhat greater than 50 residues in length (i.e., too large to be chemically synthesized). Assuming that the cDNA for the gene is available, N-terminal Cys polypeptides can be obtained using a mutagenesis/proteolysis strategy (Erlandson et al., 1996). This involves constructing an expression vector in which a DNA sequence encoding the peptide Ile-Glu-Gly-Arg-Cys is inserted between a upstream affinity-purification handle (e.g., MBP, GST, or His tag) and the appropriate gene fragment of interest (for cloning and mutagenesis see *UNIT 6.6*). Following expression and affinity purification, the fusion protein is treated with Factor Xa to give the desired recombinant Cys-polypeptide, which can then be further purified if necessary. These recombinant Cys-polypeptides can be used in native chemical ligation reactions as per their synthetic counterparts.

SUPPORT PROTOCOL 3

CHEMICAL SYNTHESIS OF α-THIOESTER POLYPEPTIDES

This protocol describes the chemical synthesis of a 3-mercaptopropionamide α-thioester polypeptide on a 3-mercaptopropionamide-MBHA resin. This resin can be easily prepared from commercially available MBHA resin using a three-step solid-phase procedure (Fig. 18.4.3). The solid-phase synthesis of the polypeptide is achieved using Boc amino acid derivatives employing in situ neutralization/HBTU [2-(1H-benzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate] activation protocols for Boc-SPPS (Schnölzer et al., 1992). The corresponding α-thioester polypeptide, suitable for native chemical ligation, is obtained after cleavage with hydrogen fluoride and purification.

Materials

Methylbenzhydrylamine (MBHA) resin (Peptides International)
Dimethylformamide (DMF, spectrophotometric grade; Fisher)
5% (v/v) diisopropylethylamine (DIEA, peptide synthesis grade; Perkin-Elmer Applied Biosystems) in DMF (store up to 1 month at room temperature)
97% 3-bromopropionic acid
Dichloromethane (DCM, spectrophotometric grade; Fisher)
99% diisopropyl carbodiimide (DIPC, 99%; Aldrich)
Diisopropylethylamine (DIEA, peptide synthesis grade; Perkin-Elmer Applied Biosystems)

continued

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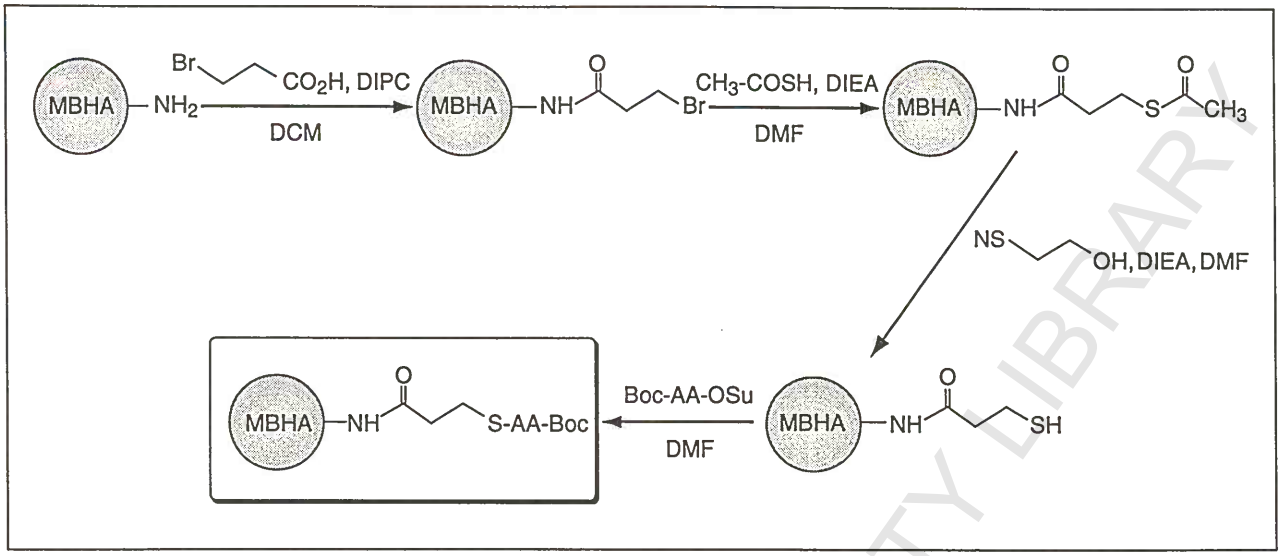

Figure 18.4.3 Scheme showing the preparation of the Boc-aminoacyl-3-mercaptopropionamide-MBHA resin using solid-phase transformations. Abbreviations: AA, aminoacyl; Boc, *t*-butyloxycarbonyl; CH_3-COSH, thioacetic acid; DCM, dichloromethane; DIEA, diisopropylethylamine; DIPC, diisopropyl carbodiimide; DMF, dimethylformamide; MBHA, methylbenzhydrylamine; OSu, hydroxysuccinimide ester.

Ac_2O/DIEA/DMF solution (see recipe)
AcSH/DIEA/DMF solution (see recipe)
BME/DIEA/DMF solution (see recipe)
Boc–amino acyl–N-hydroxysuccinimide ester (Boc-AA-OSu; where AA is the first amino acid to be incorporated into the polypeptide; Bachem)
Trifluoroacetic acid (TFA, BioGrade)
Ninhydrin test reagents: monitor 1, monitor 2, and monitor 3 (Perkin-Elmer Applied Biosystems)
HF/*p*-cresol solution (see recipe)
Diethyl ether, cold
10% to 50% acetonitrile in H_2O containing 0.1% TFA

15-ml manual peptide synthesis vessel (Peptides International)
Black rubber tubing (1/4 in. i.d. × 5/8 in. o.d. × 3/16 in. wall thickness; Fisher), resistant to acids and organic solvents
2-liter side-arm flasks with rubber stoppers and glass tubing to fit
Pasteur pipet containing glass wool for filtration
2-ml polypropylene column (Microcolumn X from Isolab) with Teflon stopcock
13 × 100–mm glass test tube
110°C heating block
60% ethanol
HF cleavage apparatus (Peptides International)

Additional material and equipment for solid-phase peptide synthesis (Schnölzer et al., 1992; *UNIT 18.1*), preparative C18 reversed-phase HPLC (see Basic Protocol 1, step 11), and ESI-MS (Chapter 16)

Prepare the MBHA resin

1. Build a manual solid-phase peptide synthesis system (Fig. 18.4.4) consisting of a 15-ml manual peptide synthesis vessel attached by 12 in. of black rubber tubing through a rubber stopper to a 2-liter side-arm flask, which is in turn connected to a vacuum source.

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2. Place 1 g of MBHA resin (1.0 mmol/g) into the peptide synthesis vessel, add enough DMF to cover the dry resin (~5 ml), and wait 30 min.

This preswelling step is crucial for the success of any reaction carried out on a solid support, since it renders the functional groups on the polymer available for reaction.

3. Drain the resin. Cover the resin with 5% DIEA/DMF, leave for 1 min, then drain the resin. Repeat this process two more times, then perform three 20-sec flow washes with DMF—i.e., wash the resin with a continuous flow of DMF for 20 sec while keeping a constant volume of solvent above the resin bed (usually 3 to 4 mm), then drain the resin.

A continuous flow wash is an extremely efficient way of exchanging solvent in a swollen polymer—much more so than the commonly used bulk wash (i.e., by adding solvent, mixing, and draining).

Prepare the symmetrical anhydride of 3-bromopropionic acid

4. Dissolve 1.222 g 3-bromopropionic acid (8 mmol) in a minimal volume of DCM (~1 to 2 ml), then add 630 µl of 99% DIPC (4 mmol). Shake the solution vigorously and wait 10 min.

A white precipitate corresponding to the diisopropyl urea will appear during the activation reaction. If no such precipitate forms, the reaction has not taken place and should be repeated.

It is best to prepare the symmetrical anhydride fresh for each coupling reaction.

5. Filter the solution through a Pasteur pipet containing glass wool.

It is very important to remove the diisopropyl urea by filtration before adding the symmetrical anhydride solution to the resin. The authors have found that if this step is not carried out the acylation reaction does not go to completion.

Couple the symmetrical anhydride of 3-bromopropionic acid to the MBHA resin

6. Add the filtered solution from step 5 to the MBHA resin in the peptide synthesis vessel. Without applying any suction, add 800 µl DIEA (4.5 mmol) and the minimal amount of DMF (1 to 2 ml) required to give a good slurry.

7. Leave the coupling reaction for 30 min with occasional stirring using a glass stirring rod.

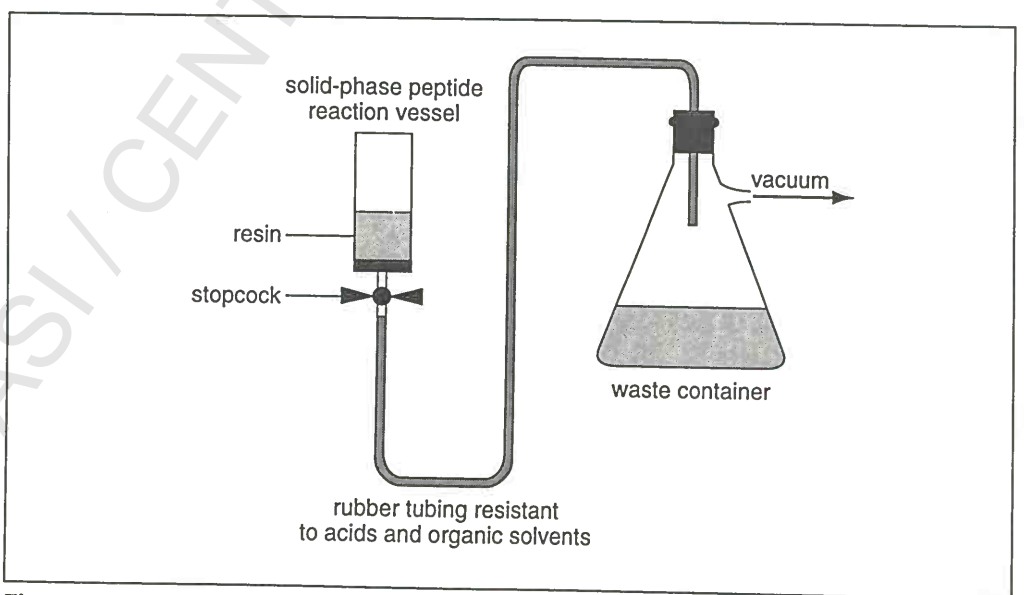

Figure 18.4.4 Suggested apparatus for the manual solid-phase synthesis of thioester peptides.

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8. Drain the resin and wash thoroughly with three 20-sec DMF flow washes as in step 3.

9. Repeat the coupling process (steps 4 to 8) two more times.

10. Add 5 ml Ac_2O/DIEA/DMF solution to the resin and wait 10 min. Drain the resin and wash three times with DMF as in step 3.

This acetylation step ensures that the small amount of unreacted amine groups remaining on the MBHA resin cannot participate in any subsequent acylation steps.

Prepare the 3-mercaptopropionamide-MBHA resin

11. Add 5 ml AcSH/DIEA/DMF solution to the resin and wait 20 min. Drain the resin and wash three times with DMF as in step 3. Repeat this entire process (treatment with AcSH/DIEA/DMF solution, along with the washings) two more times.

12. Add 5 ml BME/DIEA/DMF solution to the resin and wait 20 min. Drain the resin and wash three times with DMF as in step 3. Repeat this entire process (treatment with BME/DIEA/DMF solution, along with the washings) two more times.

The 3-mercaptopropionamide-MBHA resin which is the product of this step is susceptible to oxidation upon long-term storage. The authors therefore recommend that it be immediately acylated with the appropriate Boc amino acid derivative.

Couple the first Boc amino acid to the 3-mercaptopropionamide-MBHA resin

13. Dissolve 3 molar equivalents of the appropriate Boc-AA-OSu (where AA is the first amino acid to be incorporated in the synthesis) in ~6 ml DMF, then add this solution to the 3-mercaptopropionamide-MBHA resin. Add 714 µl DIEA (4 molar equivalents, 4 mmol) and leave the coupling reaction for 3 to 4 hr with occasional stirring.

Most of the Boc amino acid derivatives are commercially available as N-hydroxysuccinimide esters. If the required Boc amino acid derivative is not commercially available it can be readily prepared manually (Bodanszky and Bodanszky, 1994).

14. Drain the resin and wash three times with DMF as in step 3. Add 5 ml Ac_2O/DIEA/DMF solution to the resin and wait 10 min, then wash the resin three times with dichloromethane (DCM) using 20-sec flow washes as described in step 3. Dry the resin under vacuum and store at –20°C.

Determine the final substitution of the Boc-amino acyl-3-mercaptopropionamide-MBHA resin

15. Place ~5 mg of the dry Boc–amino acyl–3-mercaptopropionamide–MBHA resin from step 14 in a 2-ml polypropylene Microcolumn X column with a Teflon stopcock, connected to a vacuum source.

16. Add 1 ml TFA to the resin and wait 2 min. Drain the column, then flow wash first with DMF and then with DCM. Drain again and dry under vacuum.

17. Weigh an exact amount of the dried resin (~3 to 5 mg) and place in a 13 × 100–mm glass test tube.

18. Add 2 drops of ninhydrin test monitor 1 reagent, 4 drops of ninhydrin test monitor 2 reagent, and 2 drops of ninhydrin test monitor 3 reagent, then incubate 5 min at 110°C in a heating block.

19. Dilute the blue solution with 60% ethanol to 25 ml and mix well. Measure the absorbance at 570 nm using 1-cm path-length cuvette. Calculate the substitution of the thioester resin (in mmol/g) as $1.67 \times (A_{570}$/amount of resin in mg).

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For a resin with an initial loading of ~1 mmol/g the final substitution for the corresponding acylated thioester resin is usually ~0.2 mmol/g.

Perform solid-phase synthesis of the α-thioester polypeptide

20. Synthesize the polypeptide sequence using the in situ neutralization/HBTU activation protocols for Boc-SPPS (Schnölzer et al., 1992; *UNIT 18.1*).

    Solid-phase deprotection of the 2,6-dinitrophenyl (Dnp) and formyl (For) protecting groups (commonly used for the protection of the side chains of His and Trp residues, respectively) results in premature cleavage of α-thioester polypeptides from the resin. Therefore, these residues should either be deprotected after the ligation reaction is complete or incorporated as Boc-Trp-OH (no side-chain protection) or Boc-Trp(Hoc)-OH and Boc-His(Bom)-OH during SPPS.

21. Once the synthesis is complete, flow wash with DMF and DCM as in step 14. Dry the resin under vacuum and store at –20°C.

Cleave and purify the α-thioester polypeptide

22. Using 200 mg of peptide α-thioester resin from step 21, cleave the peptide-resin by treating with 5 ml of HF:*p*-cresol solution for 1 hr at 4°C in an HF cleavage apparatus.

    CAUTION: *Anhydrous HF is a highly toxic and corrosive gas and should only be manipulated in a fume hood using the commercially available specialized apparatus.*

23. Remove the HF under vacuum and resuspend both peptide and resin in ~40 ml cold diethyl ether with gentle stirring for 10 min. Filter the suspension on a glass Buchner funnel under vacuum, without letting air pass through the filter, as this could oxidize the cleaved peptide.

24. Wash the material in the filter (containing the cleaved peptide and the resin) three times, each time with 10 ml cold diethyl ether. Wash an additional three times, each time with 10 ml dichloromethane, then wash with 10 ml cold diethyl ether again. Discard the wash liquid.

    These washes will remove most of the scavenger byproducts.

25. Add 10 ml freshly degassed 50% acetonitrile in water containing 0.1% TFA to the filter, wait 10 min (to dissolve the cleaved peptide), then filter. Repeat this process three more times, then recover and lyophilize the filtrates.

26. Analyze a 50-µl aliquot by reversed-phase HPLC and ESI-MS (Chapter 16; also see Basic Protocol 1, step 7).

27. Purify the lyophilized propionamide α-thioester peptide by preparative C18 RP-HPLC (see Basic Protocol 1, step 11).

SUPPORT PROTOCOL 4

BACTERIAL EXPRESSION OF α-THIOESTER POLYPEPTIDES

This protocol describes the preparation of α-thioester polypeptides using bacterial expression in *E. coli*. The desired polypeptide-encoding gene fragment is first cloned into a commercially available pTYB vector (Fig. 18.4.5). Following *E. coli* transformation, soluble expression of the polypeptide-intein-CBD (chitin binding domain) fusion protein is induced and the cells are harvested and lysed. The lysate is then loaded onto a chitin column and the fusion protein affinity purified. Finally, the target α-thioester polypeptide is cleaved from the column and eluted using a buffer containing ethanethiol.

NOTE: Initial studies using an MBP-intein-CBD system indicate that the majority of amino acid residues, when located immediately before the intein N-terminal cysteine, allow both purification of fusion proteins and efficient cleavage with thiols. However, Pro,

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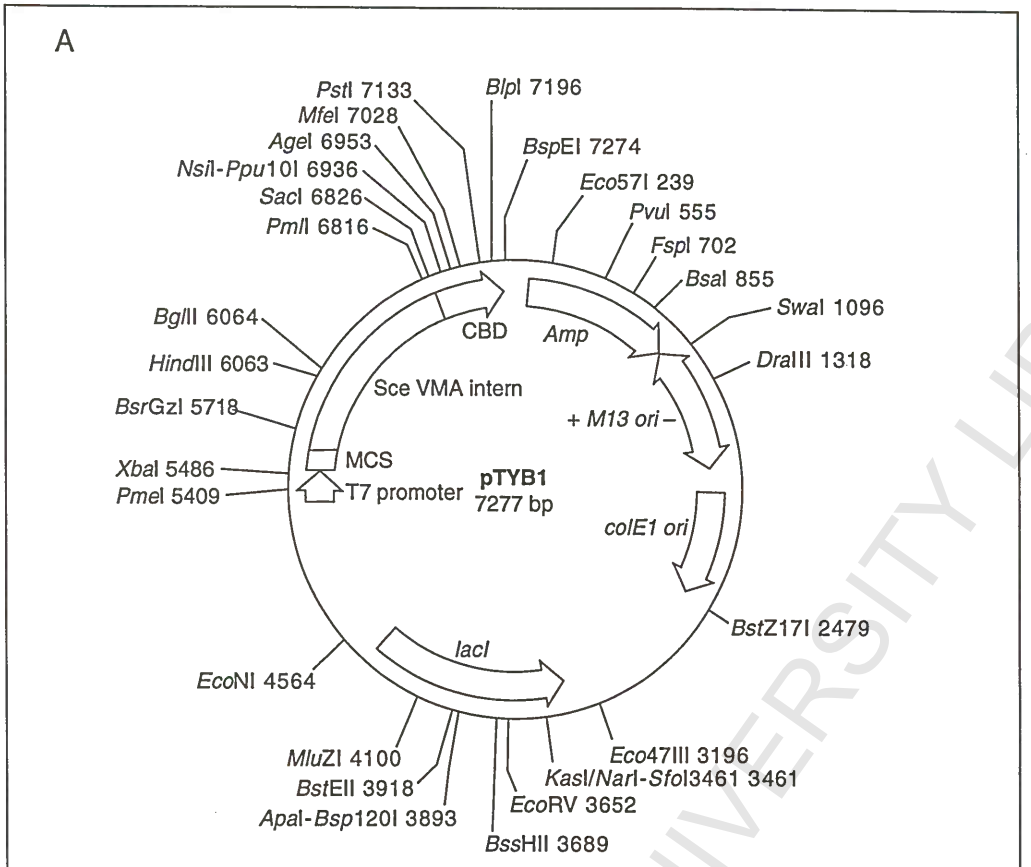

B

T7 universal primer→

5′CGG GGA TCT CGA TCC CGC GAA ATT AAT ACG ACT CAC TAT AGG GGA ATT GTG AGC

T7 promoter — *lac* operator

GGA TAA CAA TTC CCC TCT AGA AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA

*Xba*I — Shine-Dalgarno

Met Ala Ser Ser Arg Val Asp Gly Gly Arg Glu Phe Leu Glu Gly Ser Ser

CAT ATG GCT AGC TCG CGA GTC GAC GGC GGC CGC GAA TTC CTC GAG GGC TCT TCC

*Nde*I *Nhe*I *Nru*I *Sal*I *Not*I *EcoR*I *Xho*I *Sap*I

intein→

Cys

TGC TTT GCC AAG GGT ACC AAT GTT TTA ATG GCG GAT GGG TCT ATT GAA TGT

*Kpn*I

ATT GAA AAC ATT GAG GTT CGT AAT AAG GTC ATG GGT 3′

intein reverse sequencing primer

Figure 18.4.5 pTYB1, one of the series of pTYB vectors useful for the cloning, expression and purification of recombinant α-thioesters polypeptides. (**A**) pTYB1 vector; (**B**) sequence of pTYB1 cloning/expression region. This vector uses a bacteriophage T7 promoter-driven system (see *UNIT 5.1* and Ausubel et al., 1999). The target gene is cloned into the multiple cloning site (MCS) polylinker region to create an in-frame fusion between the C-terminus of the target gene and the N-terminus of the gene encoding a modified intein. The DNA encoding a small chitin binding domain (CBD) has been also added to the C-terminus of the intein gene to allow the resulting fusion protein to be purified by affinity chromatography. Following purification, the target α-thioester polypeptide is obtained through a transthioesterification reaction by treating the fusion protein with ethanethiol. The pTYB vectors are available from New England Biolabs.

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Cys, and Asn residues were found to inhibit the in vitro cleavage with thiols. Note that partial cleavage during bacterial expression can sometimes be observed in certain systems, resulting in a decrease in the yield of fusion precursors. For example, in the MBP system, Asp, Arg, His, and Glu residues gave rise to in vivo cleavage (>50%).

Materials

Gene construct encoding target polypeptide
pTYB1 vector (New England Biolabs)
E. coli BL21 (or any other suitable *E. coli* strain)
LB medium containing 100 µg/ml ampicillin (*APPENDIX 4A*)
1 M isopropyl-1-thio-β-D-galactopyranoside (IPTG), filter sterilized
Chitin beads slurry: 50% (w/v) suspension of chitin beads (New England Biolabs) in 40% ethanol
Column buffer (see recipe)
Lysis buffer (see recipe)
Cleavage buffer (see recipe)

Refrigerated centrifuge with Beckman JA-10 rotor and centrifuge buckets to hold 1 liter of culture
1.5 × 10–cm glass or polypropylene column
Refrigerated centrifuge with Beckman JA-17 rotor and appropriate centrifuge tubes
Shaker

Additional reagents and equipment for introducing plasmid vectors into bacterial cells (*APPENDIX 4D*), growth of bacteria in liquid medium (*APPENDIX 4A*), lysis of bacterial cells using a French press (*UNIT 6.3*), ESI-MS (*UNITS 16.1 & 16.2*)

Express the fusion protein

1. Insert the gene construct encoding the target polypeptide into the pTYB1 vector (see manufacturer's instruction for vector and Ausubel et al., 1999) so as to express the target protein as an intein-CBD fusion. Introduce the vector into the *E. coli* BL21 cells (see *APPENDIX 4D* and Ausubel et al., 1999). Inoculate 15 ml LB/ampicillin with *E. coli* BL21 containing a pTYB vector expressing the desired protein fusion. Grow overnight with shaking at 37°C (*APPENDIX 4A*).

2. Inoculate 1000 ml LB/ampicillin with 10 ml of the overnight culture and grow with shaking at 37°C to an OD_{600} of 0.5 to 0.6 (*APPENDIX 4A*).

3. Add 1 ml of 1 M IPTG (final concentration of 1 mM) to the culture and continue incubation for 1 to 6 hr at 37°C.

   The optimal induction conditions (i.e., incubation time, temperature, and final IPTG concentration) for soluble expression will depend on the in vivo properties of the overexpressed protein, and should be optimized for every system.

4. Centrifuge the cell culture 10 min at 8700 × *g* (7000 rpm in Beckman JA-10 rotor), 4°C. Discard supernatant. Proceed to step 5 immediately or freeze pellet at –70°C indefinitely.

   If extract preparation is to be be carried out immediately, the chitin column may be prepared during the centrifugation step.

Prepare the affinity column

5. Add ~10 ml of chitin bead slurry to a 1.5 × 10–cm column and allow liquid to drain just to the top of the packed resin bed (~5 ml).

6. Wash the column with 100 ml column buffer at a flow rate of 1 ml/min.

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Prepare the cell extract

7. If cell pellet was frozen, (step 4), thaw on ice. Resuspend pellet in 30 ml ice-cold lysis buffer. Lyse cells using a French press (*UNIT 6.3*).

   IMPORTANT NOTE: *From this step on, all procedures should be carried out at 4°C.*

8. Centrifuge lysate for 30 min (or until supernatant is clear) at 25,000 × *g* (14,000 rpm in Beckman JA-17 rotor), 4°C. Decant supernatant into a clean container on ice and discard pellet.

   The supernatant can be frozen and stored at –70°C indefinitely before continuing with the procedure.

   In order to monitor the expression, extraction, and purification steps, it is convenient to take small aliquots at every step and analyze them by SDS-PAGE (UNIT 10.1).

Purify the fusion protein

9. If extract is frozen, thaw on ice. Load onto the chitin column (at a rate no faster than 0.5 ml/min). Collect flowthrough and reapply to column, then repeat this process one more time.

   Chitin beads have a capacity of ~2 mg of CBD-tagged protein per ml packed beads. Therefore, the amount of extract that can be loaded on the column will depend on the amount of soluble fusion protein in the extract.

10. Wash the column with 100 ml column buffer at a flow rate of 1 ml/min. Discard flowthrough. Be sure that all traces of crude extract have been washed off the sides of the column.

Obtain the α-thioester polypeptide

11. Add 5 ml cleavage buffer to the column and gently shake the reaction slurry overnight at room temperature.

12. Drain the beads, then wash with 15 ml cleavage buffer (three times, each time with 5 ml). Pool all of the fractions.

    IMPORTANT NOTE: *Thioesters are susceptible to hydrolysis under alkaline conditions; consequently purification and storage of α-thioester polypeptides should be performed at pH 6.0 or below.*

Analyze and purify the α-thioester polypeptide

13. Analyze a 50-µl aliquot by reversed-phase analytical HPLC and ESI-MS (see *UNITS 16.1 & 16.2*; also see Basic Protocol 1, step 7).

14. Purify the target α-thioester polypeptide by reversed-phase preparative HPLC see Basic Protocol 1, step 11).

CHEMICAL SYNTHESIS OF N^{α}(Msc)-CYS, α-THIOESTER POLYPEPTIDES

SUPPORT PROTOCOL 5

This protocol describes how to introduce the N^{α}(methylsulfony)ethyloxycarbonyl (Msc) protecting group onto the α-amino group of an N-terminal Cys polypeptide synthesized on a 3-mercaptopropionamide-MBHA resin (see Support Protocol 3). The resulting N^{α}(Msc)-Cys, α-thioester polypeptides are used in sequential native chemical ligation reactions (see Basic Protocol 2).

NOTE: Boc-SPPS of α-thioester polypeptides requires the use of the Bom side-chain protecting group for His. The Bom group releases fomaldehyde during its deprotection with HF; however the formation of the thiazolidine adduct with the N-terminal Cys cannot take place due to the presence of the N^{α}Msc group.

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Additional Materials (also see Support Protocol 3)

Fully protected Boc-polypeptide-3-mercaptopropionamide-MBHA resin (see Support Protocol 3, step 21), dried
(Methylsulfonyl)-ethyl 4-nitrophenyl carbonate (Msc-ONp; Fluka)

Prepare the Boc-polypeptide-3-mercaptopropionamide-MBHA resin

1. Place ~0.5 g of fully protected Boc-polypeptide-3-mercaptopropionamide-MBHA resin in a peptide synthesis vessel attached to a vacuum source and swell with DMF (see Support Protocol 3, steps 1 and 2).
2. Deprotect the Boc group by adding ~5 ml of TFA to the resin, waiting 1 min, then draining the resin. Repeat this process one additional time, then drain the resin and perform three 20-sec flow washes with DMF (see Support Protocol 3, step 3).
3. Neutralize the polypeptide-3-mercaptopropionamide-MBHA resin by treating with 5% DIAE/DMF and flow washing with DMF (see Support Protocol 3, step 3).

Introduce the Msc group on the α-amino group of the polypeptide-3-mercapto-propionamide-MBHA resin

4. Dissolve 560 mg (2 mmol) of Msc-ONp in 4 to 5 ml DMF and add this mixture to the peptide thioester resin, then add 340 µl (2 mmol) of DIEA. Leave the coupling reaction for 3 hr, with occasional stirring using a glass stirring rod, then drain the resin and perform three 20-sec flow washes with DMF.
5. Place ~1 mg of resin from step 4, in a column (see Support Protocol 3, step 15). Flow wash first with DMF and then with DCM, drain again, and dry under vacuum. Place the aliquot in a glass test tube and perform the ninhydrin test (see Support Protocol 3, step 18). If the color of the solution is strongly blue, repeat the coupling reaction (step 4) and test again; if not, continue with step 6, below.

   Sometimes the ninhydrin test can give false positives (i.e., strong blue coloration) even although the acylation reaction is complete. This is especially true when the α-amino group is protected with a base-labile group (i.e., Fmoc or Msc). Therefore, if the ninhydrin test is still positive after the third coupling, proceed to step 6.

6. Perform three 20-sec flow washes with DMF and then with DCM. Dry the resin under vacuum and store at –20°C until further use.
7. Cleave and purify 200 mg of peptide-thioester resin (see Support Protocol 3, steps 22 to 26).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Ac_2O/DIEA/DMF solution

15 parts (v/v) 99% acetic anhydride (Ac_2O)
15 parts (v/v) diisopropylethylamine (DIEA, peptide synthesis grade; Perkin-Elmer Applied Biosystems)
70 parts (v/v) dimethylformamide (DMF, spectrophotometric grade; Fisher)
Prepare fresh

AcSH/DIEA/DMF solution

1 part (v/v) 96% thiolacetic acid (AcSH; Fluka)
1 part (v/v) diisopropylethylamine (DIEA, peptide synthesis grade; Perkin-Elmer Applied Biosystems)
8 parts (v/v) dimethylformamide (DMF, spectrophotometric grade; Fisher)
Prepare fresh

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BME/DIEA/DMF solution

1 part (v/v) 98% 2-mercaptoethanol (BME)
1 part (v/v) diisopropylethylamine (DIEA, peptide synthesis grade; Perkin-Elmer Applied Biosystems)
8 parts (v/v) dimethylformamide (DMF, spectrophotometric grade; Fisher)
Prepare fresh

Cleavage buffer

0.1 mM EDTA
200 mM sodium phosphate, pH 6.0
250 mM NaCl
3% (v/v) ethanethiol
0.1% (v/v) Triton X-100
Adjust pH to 6.0 with 1 M NaOH or HCl
Store up to 6 months at 4°C

Column buffer

0.1 mM EDTA
20 mM sodium phosphate, pH 7.2
250 mM NaCl
0.1% (v/v) Triton X-100
Adjust pH to 7.2 with 1 M NaOH or HCl
Store up to 6 months at 4°C

Guanidine·HCl buffer, 6 M

6 M guanidine·HCl (*APPENDIX 3A*)
0.1 M sodium phosphate
1 mM EDTA
Adjust pH to 7.5 with 1 M NaOH
Store up to 6 months at room temperature

HF/p-cresol solution

96 parts (v/v) hydrogen fluoride (HF; anhydrous)
4 parts (v/v) *p*-cresol
Prepare fresh

CAUTION: *Anhydrous HF is a highly toxic and corrosive gas and should be manipulated only in a fume hood, using the commercially available specialized apparatus (HF cleavage apparatus from Peptides International).*

Lysis buffer

0.1 mM EDTA
1 mM PMSF
25 mM HEPES, pH 8.0
250 mM NaCl
5% (v/v) glycerol
Adjust pH to 8.0 with 1 M NaOH
Store up to 6 months at 4°C

COMMENTARY

Background Information

The introduction of solid-phase peptide-synthesis (SPPS) by Bruce Merrifield revolutionized the chemical synthesis of peptides (Merrifield, 1963; also see *UNIT 18.1*). Despite the enormous impact of SPPS in the generation and study of small bioactive peptides, it is now clear that the combination of incomplete acylation/deprotection reactions and other well documented side reactions places an intrinsic limit on the size of peptides accessible by efficient stepwise SPPS. Thus, polypeptides of up

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to ~50 residues in length can be prepared by stepwise SPPS with reasonable confidence, but beyond this, the chances of success fall off precipitously. In order to overcome this size limitation, recent years have seen renewed interest in the use of convergent synthetic strategies—i.e., the synthesis of large polypeptides from smaller peptide building blocks which are themselves accessible via the SPPS approach. It should be noted that fragment condensation has a long and illustrious history in the peptide chemistry field; however, there have always been serious problems associated with the manipulation of the fully protected peptides in these classical convergent strategies. Although the use of minimal protection strategies represented a step in the right direction (see Lloyd-Williams et al., 1993 for an extensive review), it was not until the early 1990s that a truly practical way of performing fragment condensations was developed—i.e., chemical ligation (for reviews see Muir, 1995; Wallace, 1995). The original chemical ligation strategies were all based on the premise that an unnatural moiety could be used to covalently join two fully unprotected (and hence water-soluble) polypeptides, each bearing unique and mutually reactive groups. A number of different chemistries have been developed for this purpose, all of which give rise to an unnatural covalent structure at the ligation site (Muir, 1995; Wallace, 1995).

In 1994, a second-generation ligation chemistry was introduced, known as "native chemical ligation," which allows the preparation of proteins with native backbone structures from fully unprotected peptide building blocks (Dawson et al., 1994). This important extension of the chemical ligation concept makes use of the mild acylating power of the α-thioester functionality. The principle of native chemical ligation is depicted in Figure 18.4.1. The first step involves the chemoselective reaction which occurs between the free thiol group of an unprotected N-terminal Cys-polypeptide and a second, unprotected polypeptide containing an α-thioester group. This transthioesterification reaction gives rise to a thioester-linked intermediate which spontaneously rearranges to form a native peptide bond at the ligation site. The target full-length polypeptide is thus obtained without any further manipulation. Native chemical ligation reactions are performed in aqueous buffers at pH 7 to 7.5 in the presence of thiol cofactors. At this pH, the regioselectivity of the reaction is such that the reaction can to be performed in the presence of all the functionalities commonly found in proteins. Even the presence of additional Cys residues in one or both fragments does not affect the regioselectivity of the ligation (Hackeng et al., 1997). Small proteins or protein domains ~100 to 120 amino acids in length can be reliably generated from two peptide building blocks in a single chemical ligation step (for a few examples see Dawson et al., 1994, 1997; Lu et al., 1996; Hackeng et al., 1997; Camarero et al., 1998).

The total chemical synthesis of larger protein targets (>100 residues) via the ligation of just two fragments becomes more and more problematic as the size increases. This is due to the difficulties associated with the direct stepwise SPPS of polypeptide segments bigger than 50 residues. This difficulty can be overcome by performing multiple ligation reactions using three or more synthetic peptides (e.g., Canne et al., 1995). Native chemical ligation has been extended to allow multiple ligation steps to be performed sequentially in a controlled and directed way (Muir et al., 1997; Camarero et al., 1998). The general approach is depicted in Figure 18.4.2. Key to this strategy is the temporary protection of the α-amino group of the central peptide with the base-labile 2-(methylsulfonyl)ethyloxycarbonyl (Msc) group (Tesser and Balvert-Geers, 1975). The presence of this protecting group prevents the N-terminal Cys from reacting in an intramolecular or intermolecular fashion with the α-thioester functionality present in the same polypeptide fragment. Once the first ligation reaction is finished, the N^{α}-Msc group can be efficiently removed, allowing the next ligation step to be performed (Fig. 18.4.2).

The native chemical ligation approach can also be used in the semisynthesis of proteins (Muir et al., 1998; Severinov and Muir, 1998; Erlandson et al., 1996; Evans et al., 1998) from recombinant and synthetic polypeptide fragments. As described above, native chemical ligation of two polypeptides requires that one of the fragments possess an N-terminal Cys and that the other contain an α-thioester moiety. Polypeptides containing an N-terminal cysteine residue for use in litigation can be obtained using standard recombinant DNA expression methods (see Chapter 5). Importantly, biosynthetic methods are also now available for the generation of α-thioester polypeptides (Muir et al., 1998; Severinov and Muir, 1998; Evans et al., 1998). This is made possible using the IMPACT expression system, commercially available from New England Biolabs (Chong

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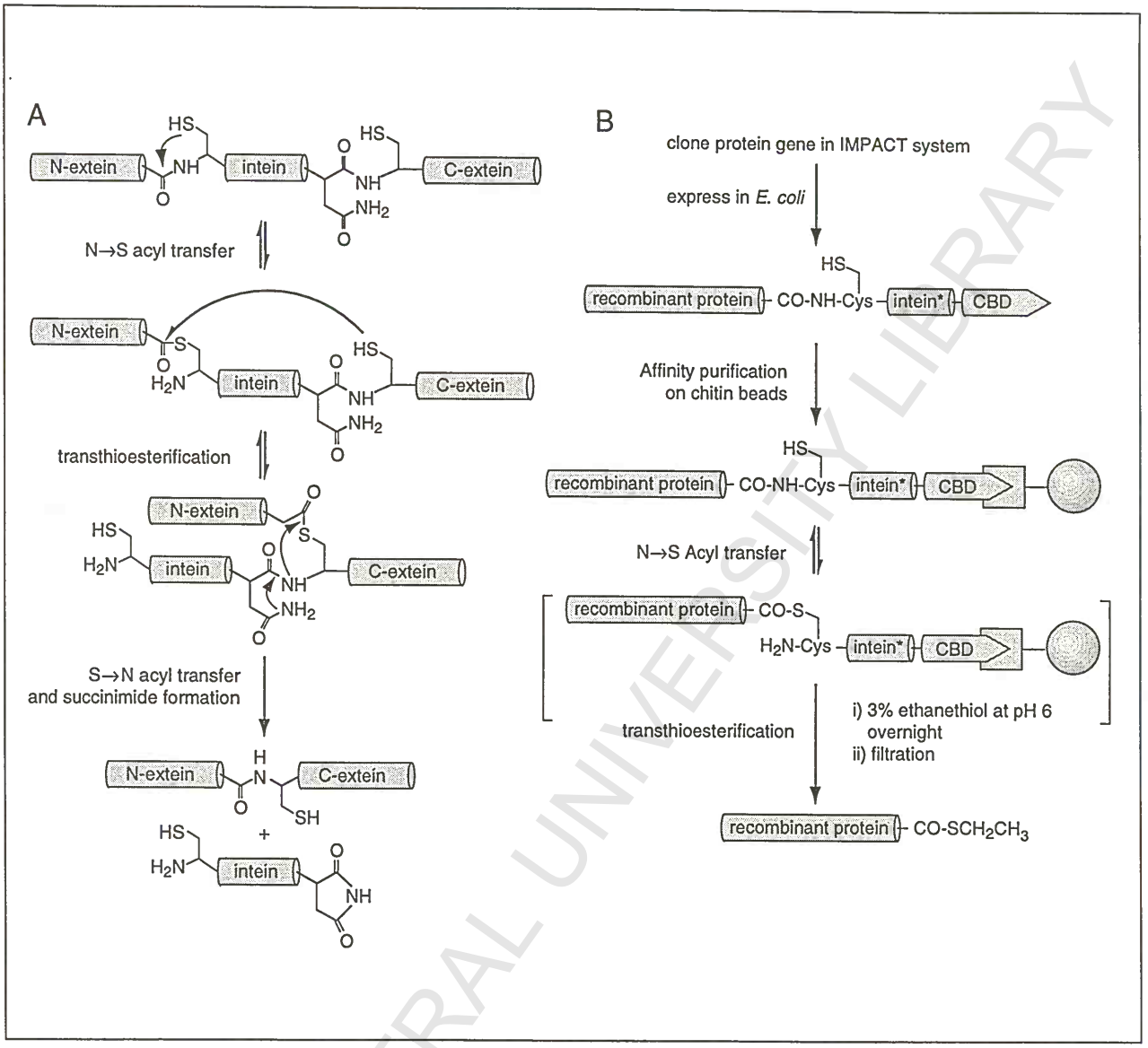

Figure 18.4.6 Principles of the biosynthetic preparation of α-thioester polypeptides by recombinant techniques. (**A**) Scheme representing the proposed mechanism of protein splicing involving the intein from *Saccharomyces cerevisiae* VMA1 gene (Xu and Perler, 1996). (**B**) Expression, purification, and cleavage of polypeptide-intein*-CBD fusion protein (where the asterisk refers to the mutation Asn454→Ala in the intein element and CBD refers to the chitin binding domain) with an appropriate thiol to give the α-thioester polypeptide.

et al., 1997). This system utilizes a protein splicing element, an intein from the *Saccharomyces cerevisiae* VMA1 gene, in conjunction with a chitin binding domain (CBD) which allows purification by affinity chromatography (Fig. 18.4.6). The natural intein has been modified in the expression system (Asn454→Ala) in order to block the normal protein splicing reaction in midstream. This results in the formation of a thioester linkage between the polypeptide of interest and the intein (Fig. 18.4.6). Cleavage of the thioester can thus be induced by treatment of the expressed fusion protein with the appropriate thiol to give the target α-thioester polypeptide (Muir et al., 1998; Severinov and Muir, 1998; Evans et al., 1998).

Critical Parameters and Troubleshooting

As mentioned in Basic Protocol 1, pH is the most critical parameter in the native chemical ligation of two fully unprotected peptides in aqueous buffers. The efficiency of the ligation reaction is strongly dependent on the pH; above pH 8.0 the reaction loses its regioselectivity and below pH 6.0 the reaction is usually very slow.

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Another crucial factor is the concentration of the two reactants. The bimolecular nature of native chemical ligation (and ligations in general) means that the concentration of both reactants should be as high as possible for efficient reaction. Generally the use of chemical denaturants (GdmCl or urea) will allow high concentrations of both reactants to be achieved. Furthermore, the use of denaturing conditions helps to alleviate potential steric problems that may be associated with the use of folded polypeptides.

Another important parameter for the success of the ligation reaction is the availability of the thiol and/or α-amino groups of the N-terminal Cys polypeptide during the ligation reaction. If one or both groups are chemically blocked, the ligation will not take place. It is well known that N-terminal Cys peptides can react rapidly with carbonyl-containing compounds (e.g., acetone and formaldehyde) to give the corresponding N-terminal thiazolidine adducts, which are totally unreactive in the native ligation process. It is thus crucial to avoid the use of these substances while handling all peptides and buffers. Note that the authors have found acetone, a commonly used solvent for washing glassware in many laboratories, to be particularly problematic in this regard.

Anticipated Results

Native chemical ligation has been applied to the synthesis and semisynthesis of a large number of proteins and protein domains. These studies indicate that very high yields (80% or better) are typically obtained for the ligation step. Following purification, the total yield usually drops to 50% to 60%. In sequential ligations where the N^{α}-Msc group has to be deprotected in situ after the first ligation step, the typical total yield after purification is ~30% to 40%. It is also important to note that the protein-folding step may further decrease the final yield of product.

Time Considerations

In Basic Protocols 1 and 2, the chemical ligation step typically requires 2 days, although in some cases the ligation reaction can proceed very rapidly (in a few hours) or somewhat more slowly (in 4 days). The purification step (RP-HPLC or other liquid chromatographies) can be performed in half a day. Processing of the purified samples (lyophilization or concentration) can take 1 to 2 days (depending on the volume). Finally, folding of the protein (if necessary) can be achieved in 3 days.

The thioester resin can be prepared in 1 day. The time required for the chemical synthesis of an α-thioester polypeptide (see Support Protocol 3) will depend on its size. Typically, for peptides ~50 residues in length, the solid-phase chain assembly can be carried out in 3 days manually or in 1 day using an automated synthesizer. The deprotection-cleavage and purification can be performed in 1 day, and lyophilization of peptide fractions can take 1 to 2 days (depending on the volume).

Bacterial expression of a peptide-intein-CBD fusion protein and preparation of the crude cell extract requires 2 days for the system described here (see Support Protocol 4). Column preparation requires ~2 to 3 hr. Loading and washing the column can be done in 3 hr. The cleavage of the α-thioester polypeptide from the affinity column requires 10 to 15 hr (overnight). Purification and processing of the polypeptide fractions requires 1 to 2 days.

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IDENTIFICATION OF PROTEIN INTERACTIONS

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Table 19.2.1 Interaction Trap Components[a,b], continued

Plasmid name/source	Selection		Comment/description
	In yeast	In *E. coli*	
pMW111[f]	*URA3*	Km^r	Same as pRB1840, but with altered antibiotic resistance marker
pMW107[f]	*URA3*	Cm^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW108[f]	*URA3*	Cm^r	Same as pJK103, but with altered antibiotic resistance marker
pMW110[f]	*URA3*	Cm^r	Same as pRB1840, but with altered antibiotic resistance marker
pJK101[e,f] (control)	*URA3*	Ap^r	Contains a *GAL1* upstream activating sequence followed by two *lexA* operators followed by *lacZ* gene; used in repression assay to assess bait binding to operator sequences

[a]All plasmids contain a 2μm origin for maintenance in yeast, as well as a bacterial origin of replication, except where noted (pEE202I, pJG4.5I).

[b]Interaction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Plasmids with altered antibiotic resistance markers (all pMW plasmids) were constructed at Glaxo in Research Triangle Park, N.C. (Watson et al., 1996). Plasmids and strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (pEG202); J. Kamens, BASF, Worcester, Mass. (pJK202); cumulative efforts of I. York, Dana-Farber Cancer Center, Boston, Mass. and M. Sainz and S. Nottwehr, U. Oregon (pNLexA); D.A. Shaywitz, MIT Center for Cancer Research, Cambridge, Mass. (pGilda); R. Buckholz, Glaxo, Research Triangle Park, N.C. (pEE2021, pJG4-51); J. Gyuris, Mitotix, Cambridge, Mass. (pJG4-5); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH17-4); R.L. Finley, Wayne State University School of Medicine, Detroit, Mich. (pRFHM1); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH18-34); J. Kamens, BASF, Worcester, Mass. (pJK101, pJK103); R. Brent, The Molecular Sciences Institute, Berkeley, California (pRB1840). Specialized plasmids not yet commercially available can be obtained by contacting the Brent laboratory at (510) 647-0690 or *brent@molsci.org* or the Golemis laboratory, (215) 728-2860 or *EA_Golemis@fccc.edu*.

[c]Sequence data are available for pEG202 (pLexA) accession number pending.

[d]Plasmids commercially available from Clontech and OriGene; for Clontech pEG202 is listed as pLexA, pJG4-5 as pB42AD, and pSH18-34 as p8op-LacZ.

[e]Plasmids and strains available from OriGene.

[f]In pMW plasmids the ampicillin resistance gene (Ap^r) is replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r) from pBC SK(+) and pBK-CMV (Stratagene), respectively. The choice between Km^r and Cm^r or Ap^r plasmids is a matter of personal taste; use of basic Ap^r plasmids is described in the basic protocols. Use of the more recently developed reagents would facilitate the purification of library plasmid in later steps by eliminating the need for passage through KC8 bacteria, with substantial saving of time and effort. Ap^r has been maintained as marker of choice for the library plasmid because of the existence of multiple libraries already possessing this marker. These plasmids are the basic set of plasmids recommended for use.

[g]Plasmids commercially available from Invitrogen as components of a Hybrid Hunter kit; this kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

porter—the EGY48 strain (or related strain EGY191) that expresses the LexA fusion protein should not grow on medium lacking Leu, and the colonies should be white on medium containing Xgal. The characterized bait protein plasmid is used for Basic Protocol 2 to screen a library for interacting proteins.

Materials

DNA encoding the protein of interest

Plasmids (see Table 19.2.1): pEG202 (see Fig. 19.2.3), pSH18-34 (see Fig. 19.2.4), pSH17-4, pRFHM1, and pJK101 for basic characterization; other plasmids for specific circumstances as described (Clontech, Invitrogen, OriGene, or R. Brent)

Yeast strain EGY48 (*ura3 trp1 his3 3LexA*-operator-*LEU2*), or EGY191 (*ura3 trp1 his3 1LexA*-operator-*LEU2*; Table 19.2.2)

Complete minimal (CM) medium dropout plates (*APPENDIX 4L*), supplemented with 2% (w/v) of the indicated sugars (glucose or galactose), in 100-mm plates:
- Glu/CM, –Ura, –His
- Gal/CM, –Ura, –His
- Gal/CM, –Ura, –His, –Leu

Z buffer (*APPENDIX 4L*) with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Xgal)

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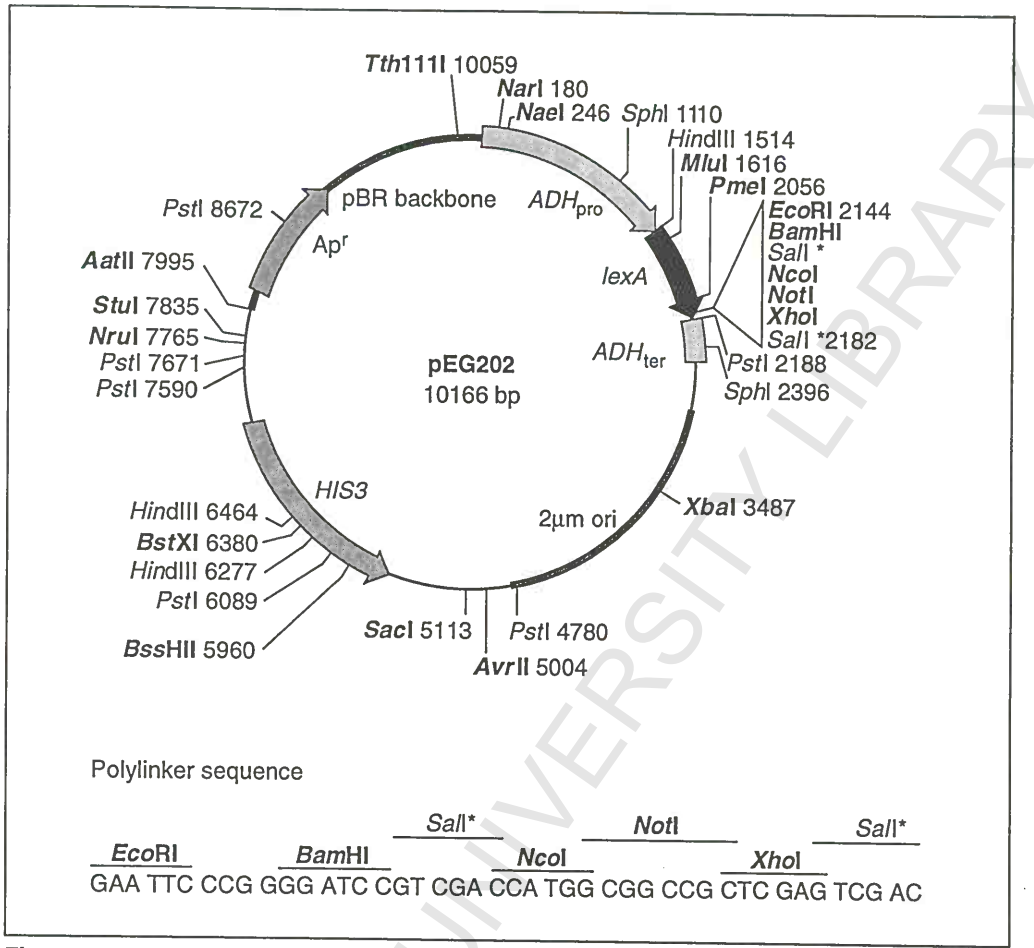

Figure 19.2.3 LexA-fusion plasmids: pEG202. The strong constitutive *ADH* promoter is used to express bait proteins as fusions to the DNA-binding protein LexA. Restriction sites shown in this map are based on recently compiled pEG202 sequence data and include selected sites suitable for diagnostic restriction endonuclease digests. A number of restriction sites are available for insertion of coding sequences to produce protein fusions with LexA; the polylinker sequence and reading frame relative to LexA are shown below the map with unique sites marked in bold type. The sequence 5′-CGT CAG CAG AGC TTC ACC ATT G-3′ can be used to design a primer to confirm correct reading frame for LexA fusions. Plasmids contain the *HIS3* selectable marker and the 2µm origin of replication to allow propagation in yeast, and the Ap^r antibiotic resistance gene and the pBR origin of replication to allow propagation in *E. coli*. In the recently developed LexA-expression plasmids pMW101 and pMW103, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r), respectively (see Table 19.2.1 for details).

Gal/CM dropout liquid medium (*APPENDIX 4L*) supplemented with 2% Gal

Antibody to LexA or fusion domain: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

H_2O, sterile

30°C incubator

Nylon membrane

Whatman 3MM filter paper

Additional reagents and equipment for subcloning DNA fragments (Struhl, 1987), lithium acetate transformation of yeast (*APPENDIX 4L*), liquid assay for β-galactosidase (*APPENDIX 4L*), preparation of protein extracts for immunoblot analysis (see Support Protocol 1), and immunoblotting and immunodetection (*UNIT 10.10*)

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Table 19.2.3 Libraries Compatible with the Interaction Trap System[a], continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[b]	Contact information
Human ovary	JG	4.6×10^6	(>1.3 kb)	OriGene
Human ovary	JG	3.5×10^6	0.5-4.0 kb (1.8 kb)	Clontech
Human heart	JG	3.0×10^6	0.3-3.5 kb (1.3 kb)	Clontech
Human placenta	Y	4.8×10^6	0.3-1.2 kb	Invitrogen
Human placenta	JG	3.5×10^6	0.3-4.0 kb (1.2 kb)	Clontech
Human mammary gland	JG	3.5×10^6	0.5-5 kb (1.6 kb)	Clontech
Human peripheral blood leucocyte	JG	1.0×10^7	(>1.3 kb)	OriGene
Human kidney	JG	3.5×10^6	0.4-4.5 kb (1.6 kb)	Clontech
Human fetal kidney	JG	3.0×10^6	(>1 kb)	OriGene
Human spleen	Y	1.14×10^7	0.4-1.2 kb	Invitrogen
Human prostate	Y	5.5×10^6	0.4-1.2 kb	Invitrogen
Human normal prostate	JG	1.4×10^6	0.4-4.5 kb (1.7 kb)	Clontech
Human prostate	JG	1.4×10^6	(>1 kb)	OriGene
Human prostate cancer	JG	1.1×10^6	(>0.9 kb)	OriGene
Human fetal prostate	JG	—	—	OriGene
Human fetal liver	JG	3.5×10^6	0.3-4.5 kb (1.3 kb)	Clontech
Human fetal liver	Y	2.37×10^6	0.3-1.2 kb	Invitrogen
Human fetal liver	JG	8.6×10^6	(>1 kb)	OriGene
Human fetal brain	JG	3.5×10^6	0.5-1.2 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
Mouse brain	JG	6.1×10^6	(>1 kb)	OriGene
Mouse brain	JG	4.5×10^6	0.4-4.5 kb (1.2 kb)	Clontech
Mouse breast, lactating	JG	1.0×10^7	0.4-3.1 kb	OriGene
Mouse breast, involuting	JG	1.0×10^7	0.4-7.0 kb	OriGene
Mouse breast, virgin	JG	1.0×10^7	0.4-5.5 kb	OriGene
Mouse breast, 12 days pregnant	JG	6.3×10^6	0.4-5.3 kb	OriGene
Mouse skeletal muscle	JG	7.2×10^6	0.4-3.5 kb	OriGene
Rat adipocyte, 9-week-old Zucker rat	JG	1.0×10^7	0.4-5.0 kb	OriGene
Rat brain	JG	4.5×10^6	0.3-3.4 kb	OriGene
Rat brain (day 18)	JG	—	—	H. Niu
Rat testis	JG	8.0×10^6	(>1.2 kb)	OriGene
Rat thymus	JG	8.2×10^6	(>1.3 kb)	OriGene
Mouse liver	JG	9.5×10^6	(>1.4 kb)	OriGene
Mouse spleen	JG	1.0×10^7	(>1 kb)	OriGene
Mouse ovary	JG	4.0×10^6	(>1.2 kb)	OriGene
Mouse prostate	JG	—	—	OriGene
Mouse embryo, whole (19-day)	JG	1.0×10^5	0.2-2.5 kb	OriGene
Mouse embryo	JG	3.6×10^6	0.5-5 kb (1.7 kb)	Clontech
Drosophila melanogaster, adult, cDNA	JG	1.8×10^6	(>1.0 kb)	OriGene
D. melanogaster, embryo, cDNA	JG	3.0×10^6	0.5-3.0 kb (1.4 kb)	Clontech
D. melanogaster, 0-12 hr embryos, cDNA	JG	4.2×10^6	0.5-2.5 kb (1.0 kb)	R. Brent
D. melanogaster, ovary, cDNA	JG	3.2×10^6	0.3-1.5 kb (800 bp)	R. Brent
D. melanogaster, disc, cDNA	JG	4.0×10^6	0.3-2.1 kb (900 bp)	R. Brent
D. melanogaster, head	JG	—	—	M. Rosbash

continued

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Table 19.2.3 Libraries Compatible with the Interaction Trap System[a], continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[b]	Contact information
Miscellaneous				
Synthetic aptamers	PJM-1	$>1\times 10^9$	60 bp	R. Brent
Saccharomyces cerevisiae, S288C, genomic	JG	$>3\times 10^6$	0.8-4.0 kb	R. Brent
S. cerevisiae, S288C, genomic	JG	4.0×10^6	0.5-4.0 kb	OriGene
Sea urchin ovary	JG	3.5×10^6	(1.7 kb)	Clontech
Caenorhabditis elegans	JG	3.8×10^6	(>1.2 kb)	OriGene
Agrobacterium tumefaciens	JG	—	—	—
Arabidopsis thaliana, 7-day-old seedlings	JG	—	—	H.M. Goodman
Tomato (*Lycopersicon esculentum*)	JG	8×10^6	—	G.B. Martin
Xenopus laevis embryo	JG	2.2×10^6	0.3-4 kb (1.0 kb)	Clontech

[a]Most libraries are constructed in either the pJG4-5 vector or the pYESTrp vector (JG or Y in the Vector column); the peptide aptamer library is made in the pJM-1 vector. Libraries available from the public domain were constructed by the following individuals: (1) J. Gyuris; (3) C. Sardet and J. Gyuris; (4) W. Kolanus, J. Gyuris, and B. Seed; (39) D. Krainc; (50-52) R. Finley; (55) P. Watt; (54) P. Colas, B. Cohen, T. Jessen, I. Grishina, J. McCoy, and R. Brent (Colas et al., 1996). All libraries mentioned above were constructed in conjunction with and are available from the laboratory of Roger Brent, (510) 647-0690 or *brent@molsci.org*. The following individual investigators must be contacted directly: (18) J. Pugh, Fox Chase Cancer Center, Philadelphia, Pa.; (8,9) Vinyaka Prasad, Albert Einstein Medical Center New York, N.Y.; (57, 58) Gregory B. Martin, *gmartin@dept.agry.purdue.edu*; (11) Huifeng Niu, *hn34@columbia.edu*; (16) Christer Hoog, *christer.hoog@cmb.ki.se*; (12) Kurt Gustin, *kgus@umich.edu*; (6) Stephan Witte, *Stephan.Witte@nimbus.rz.uni-konstanz.de*.

[b]Insert size ranges for pJG4-5 based libraries originally constructed in the Brent laboratory, which are now commercially available from Clontech, were reestimated by the company.

Transform the library

1. Grow an ~20-ml culture of EGY48 or EGY191 containing a *LexA*-operator-*lacZ* reporter plasmid and pBait in Glu/CM –Ura, –His liquid dropout medium overnight at 30°C.

   For best results, the pBait and lacZ reporter plasmids should have been transformed into the yeast within ~7 to 10 days of commencing a screen.

2. In the morning, dilute culture into 300 ml Glu/CM –Ura, –His liquid dropout medium to 2×10^6 cell/ml (OD_{600} = ~0.10). Incubate at 30°C until the culture contains ~1×10^7 cells/ml (OD_{600} = ~0.50).

3. Centrifuge 5 min at 1000 to 1500 × *g* in a low-speed centrifuge at room temperature to harvest cells. Resuspend in 30 ml sterile water and transfer to 50-ml conical tube.

4. Centrifuge 5 min at 1000 to 1500 × *g*. Decant supernatant and resuspend cells in 1.5 ml TE buffer/0.1 M lithium acetate.

5. Add 1 µg library DNA in pJG4-5 and 50 µg high-quality sheared salmon sperm carrier DNA to each of 30 sterile 1.5-ml microcentrifuge tubes. Add 50 µl of the resuspended yeast solution from step 4 to each tube.

   The total volume of library and salmon sperm DNA added should be <20 µl and preferably <10 µl.

   A typical library transformation will result in 2 to 3 × 10^6 primary transformants. Assuming a transformation efficiency of 10^5/µg library DNA, this transformation requires a total of 20 to 30 µg library DNA and 1 to 2 mg carrier DNA. Doing transformations in small aliquots helps reduce the likelihood of contamination, and for reasons that are not clear, provides significantly better transformation efficiency than scaled-up versions.

   Do not use excess transforming library DNA per aliquot of competent yeast cells because each competent cell may take up multiple library plasmids, complicating subsequent analysis.

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Phage-Based Expression Cloning to Identify Interacting Proteins

Interaction cloning (also known as expression cloning) is a technique to identify and clone genes which encode proteins that interact with a protein of interest, or "bait" protein. Phage-based interaction cloning requires a gene encoding the bait protein and an appropriate expression library constructed in a bacteriophage expression vector, such as λgt11. The gene encoding the bait protein is used to produce recombinant fusion protein in *E. coli*. The cDNA is radioactively labeled with ^{32}P. A recognition site for cyclic adenosine 3′, 5′-phosphate (cAMP)–dependent protein kinase (protein kinase A; PKA) is introduced into the recombinant fusion protein to allow its enzymatic phosphorylation by PKA and [γ-^{32}P]ATP. The procedure presented here (see Basic Protocol) involves a fusion protein consisting of bait protein and glutathione-*S*-transferase (GST) with a PKA site at the junction between them (the protocol can, however, be adapted to use other PKA-containing recombinant proteins). The labeled protein is subsequently used as a probe to screen a λ bacteriophage-derived cDNA expression library, which expresses β-galactosidase fusion proteins that contain in-frame gene fusions. The phages lyse cells, form plaques, and release fusion proteins that are adsorbed onto nitrocellulose membrane filters. The filters are blocked with excess nonspecific protein to eliminate nonspecific binding and probed with the radiolabeled bait protein (see Fig. 19.3.1). This procedure leads directly to the isolation of genes encoding the interacting protein, bypassing the need for purification and microsequencing or for antibody production.

NOTE: Radioactive label is used in this protocol, and appropriate precautions and shielding should be used (*APPENDIX 2B*).

STRATEGIC PLANNING

There are two important choices one must make to begin this procedure: (1) how to design the bait protein and (2) how to construct or acquire an appropriate phage-derived expression library.

Vectors for recombinant fusion protein expression that contain a PKA recognition site can be obtained commercially. Several companies now sell these vectors with various affinity tags such as GST (Pharmacia Biotech), histidine (Novagen), or calmodulin-binding protein (Stratagene). Alternatively, one can engineer the PKA recognition site (the five–amino acid sequence RRASV) into existing vectors by using synthetic DNA that encodes it.

Lambda-derived expression libraries that direct the expression of cDNAs made from many different mRNA sources are widely available. Alternatively, a library may be constructed for a particular experimental purpose; when designing a library to be used in expression cloning, several points should be considered. Libraries made from cDNA synthesized with random primers or an oligo dT-primer during first-strand synthesis (Klickstein et al., 1995; Klickstein and Neve, 1991) can often be advantageous in that multiple clones representing different portions of the same protein can be identified in the screening. Analysis of coding regions present in these multiple clones could provide useful information about what region of the protein is responsible for the observed interaction. Full-length cDNA clones can subsequently be obtained from other available libraries.

Many suitable λ vectors are available for constructing cDNA expression libraries. The most widely used cDNA expression vector has been λgt11 (Huynh et al., 1985). There are modifications of these λ vectors, however, that facilitate the recovery of the cDNA

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inserts by avoiding the necessity of time-consuming λ phage DNA preparations. These modified vectors include those that make use of the *Cre-lox* recombination for in vivo conversion of recombinant phages into plasmid DNA in *Cre* recombinase-expressing host strains (e.g., λZipLox, Life Technologies) as well as those that employ helper phage for in vivo excision of a phagemid vector (e.g., the λZAP series of vectors, Stratagene). Most λ vectors available produce fusions of the cDNA inserts to β-galactosidase, because they are cloned into the *lacZ* gene. However, some λ-derived expression vectors (e.g., λSCREEN-1, Novagen) direct expression of proteins fused to the T7 DNA polymerase promoter (gene *10* under control of the T7 promoter) and may yield higher expression of the library proteins (Margolis et al., 1992).

BASIC PROTOCOL

INTERACTION CLONING

Phage-based interaction expression cloning is a simple, rapid, and powerful technique to identify interacting proteins. A protein of interest is expressed as a recombinant fusion protein and labeled with ^{32}P at a serine residue in an engineered PKA recognition site to facilitate detection. β-galactosidase proteins that are fused in-frame to cDNA inserts in a bacteriophage λ-derived expression library are produced by the phage and adsorbed onto nitrocellulose filters. The filters are then screened with the radiolabeled protein probe to identify phage clones that express an interacting protein.

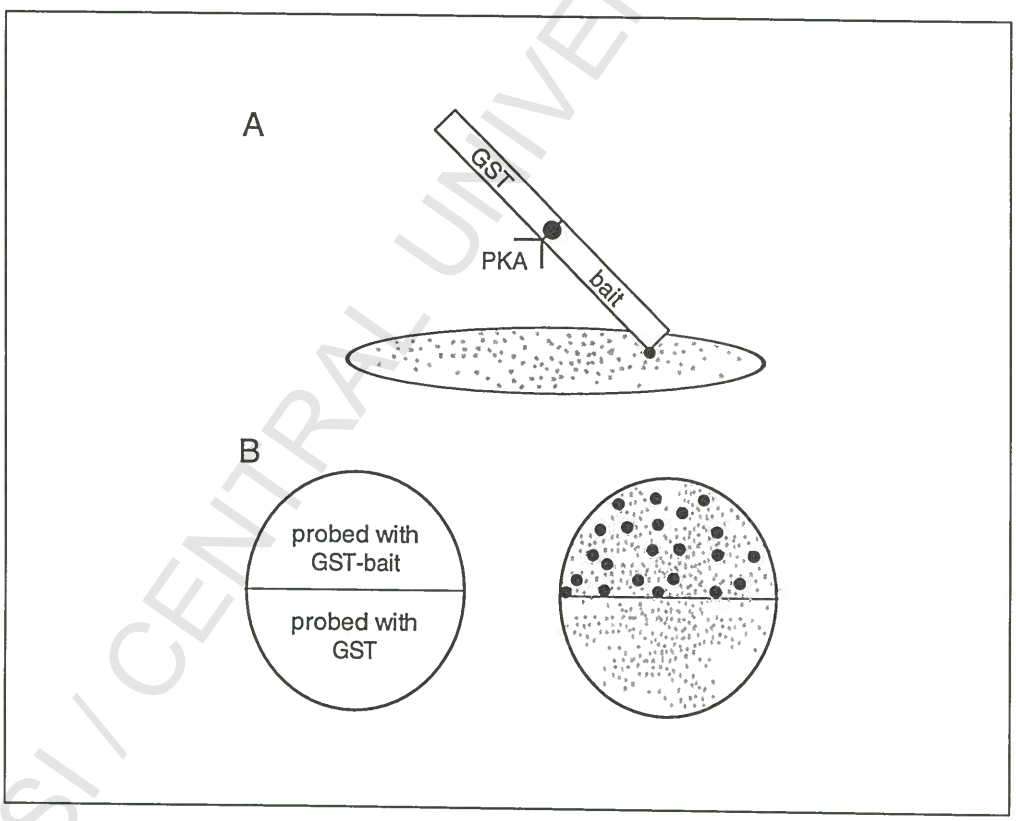

Figure 19.3.1 Schematic representation of the interaction cloning technique used to identify proteins that associate with a protein of interest (bait protein), and the expected results of a successful screen. (**A**) Expression of β-galactosidase fusion proteins from in-frame cDNA inserts of a λgt11 *Arabidopsis* library is induced with IPTG-impregnated nitrocellulose membrane filters (indicated by an oval). Filters are probed with GST-bait fusion protein labeled with ^{32}P (•) at the PKA recognition site located at the junction of the fusion. Interacting clones are detected by autoradiography. (**B**) Representative autoradiogram of a tertiary screen of a positive plaque after the control experiment to determine whether the interaction is specific for the bait portion of the probe. The top half of the filter is probed with GST-bait while the bottom half is probed with GST alone as a control.

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Materials

cAMP-dependent protein kinase (PKA; e.g., 250-U lots from Sigma)
40 mM DTT, prepared fresh
10× PKA buffer (see recipe)
10 mCi/ml [γ-^{32}P]ATP (6000 mCi/mmol)
Purified glutathione-*S*-transferase (GST)–bait protein fusion protein with a PKA recognition site (*UNIT 6.6*), at ~0.1 to 1 µg/µl concentration
Z′-KCl (see recipe), ice cold
Sephadex G-50 equilibrated in Z′-KCl
E. coli Y1090r$^-$ or other appropriate host strain
LB medium containing appropriate selective antibiotic (*APPENDIX 4B*), 10 mM $MgSO_4$, and 0.2% maltose
10 mM $MgSO_4$
10 mM IPTG (Table 1.4.2)
150- or 100-mm LB plates (with antibiotic, if necessary; *APPENDIX 4B*)
0.7% top agarose (*APPENDIX 4B*), 47°C
Tris-buffered saline with Triton X-100 (TBS-T; see recipe)
India ink
HEPES blocking buffer (HBB; see recipe)
Binding buffer (BB; see recipe)
Suspension medium (SM; see recipe)
Chloroform

3-ml disposable plastic columns *or* disposable syringe and glass wool
Scintillation counter
Tabletop centrifuge or equivalent
Nitrocellulose membrane filters (137- and 82-mm disks)
22-G needle

Additional reagents and equipment for preparation and purification of recombinant glutathione-*S*-transferase fusion protein (*UNIT 6.6*), SDS-PAGE (optional; *UNIT 10.1*), autoradiography (*UNIT 10.11*), titering and plating λ phage to generate plaques (Lech and Brent, 1988; Quertermous, 1996), and purification of bacteriophage clones (Quertermous, 1987)

Prepare the ^{32}P-labeled protein probe

1. Resuspend 250 U PKA in 25 µl freshly prepared 40 mM DTT. Let the reconstituted enzyme stand at room temperature ~10 min before use.

   It is important to use freshly prepared 40 mM DTT. PKA is extremely unstable after reconstitution; the enzyme stock solution can be stored temporarily at 4°C but retains activity for only 2 to 3 days.

2. Prepare a phosphorylation reaction mixture containing:

   1 µl 10 U/µl PKA (from step 1)
   3 µl 10× PKA buffer
   5 µl 10 mCi/ml (6000 mCi/mmol) [γ-^{32}P]ATP
   1 to 10 µl (~1 µg) purified GST-bait fusion protein
   H_2O to 30 µl.

   Incubate 1 hr at room temperature.

   A fusion protein unrelated to the bait protein, but containing the PKA recognition site, should also be expressed for use as a control to determine whether the observed interaction is specific for the bait moiety. Due to the instability of PKA, it is advisable to label both the bait protein and the unrelated control fusion protein simultaneously (in separate reactions).

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3. Add 170 µl ice-cold Z′-KCl (to stop reaction) and store on ice until use.

4. Prepare a gel filtration column by pouring Sephadex G-50 equilibrated in Z′-KCl into a 3-ml disposable plastic column (or a 3-ml syringe with a glass wool plug) for a final bed volume of ~3 ml. Allow the column to drain until the level of the buffer is at the top of the column bed.

   The Sephadex G-50 can be swelled in water or buffer and stored at 4°C for months. In this case, five to ten column volumes of Z′-KCl can be used to equilibrate the column after it is poured.

5. Load the entire phosphorylation reaction (from step 3) onto the column and collect the effluent in a 1.5-ml microcentrifuge tube. Place a second tube under the column, add a 200-µl aliquot of Z′-KCl, and collect the effluent again. Repeat the above loading and collecting steps an additional ten times to collect a total of twelve 200-µl fractions.

6. Measure the Cerenkov counts with a scintillation counter to identify the fractions with the highest specific activities, and calculate cpm/µl.

   Typically two peaks of radioactivity are observed. The first peak elutes in fractions five to nine and corresponds to labeled protein. The second peak, usually found in the last few fractions collected, corresponds to unincorporated ATP and should be discarded. The hottest fraction(s), which elute first, should be used. Fractions can be stored at 4°C for several weeks.

7. *Optional:* Analyze a small amount (1 to 2 µl) of the ^{32}P-labeled protein probe by SDS-PAGE and autoradiography (*UNITS 10.1 & 10.11*).

   Typically two strong signals are observed: one at the predicted size of the GST-bait fusion protein and the other at the predicted molecular weight of the GST alone (28 kDa). This is due to the fusion protein's inherent susceptibility to protease cleavage at the GST-bait protein junction. The presence of labeled GST protein in the probe should not interfere with the screen; a control experiment with labeled GST will be performed at the later stages of screening.

Prepare host strain cells and dilution of bacteriophage cDNA library

8. Using serial dilution, determine the titer of the bacteriophage cDNA library.

   See Lech and Brent (1988) for serial dilution procedure.

9. Grow an overnight 50-ml culture of *E. coli* Y1090r$^-$ (or other appropriate host strain; *APPENDIX 4B*) in LB medium containing an appropriate selective antibiotic, 10 mM $MgSO_4$, and 0.2% maltose.

10. Centrifuge cells 10 min at 2000 × *g*, room temperature, and resuspend in 25 ml of 10 mM $MgSO_4$.

    The resuspended cells can be stored up to 1 week at 4°C before use.

Prepare the filters to screen the bacteriophage cDNA expression library

11. Soak eight 137-mm nitrocellulose filters in 10 mM IPTG for 15 min at room temperature and air dry.

    IPTG-impregnated filters can be stored in a petri plate until use.

12. Prepare eight 1.5-ml microcentrifuge tubes each containing 0.6 ml Y1090r$^-$ cells (from step 10) and ~40,000 pfu bacteriophage (from step 8), and incubate 15 min at 37°C.

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13. Add contents of each tube to 7 ml of 0.7% top agarose at 47°C and pour onto 150-mm LB plates (with antibiotic, if necessary). Incubate plates ~3 hr at 42°C, until small plaques are visible.

14. Overlay the plates with IPTG-impregnated filters and incubate an additional 6 to 8 hr at 37°C.

    Formation of plaques in the absence of induction of the lacZ gene promoter ensures that any library-encoded proteins that are deleterious to phage growth will not be expressed while the phage are forming a plaque.

    Incubation in the presence of IPTG-impregnated filters is usually performed for a 6- to 8-hr period, but can proceed overnight for convenience.

15. Chill plates 15 min (or overnight) at 4°C.

16. Pierce each filter in several locations with a 22-G needle dipped in India ink to mark orientation. Remove filters from plates and wash 15 min in TBS-T at room temperature with shaking.

Screen bacteriophage cDNA expression library

17. Incubate filters 1 to 4 hr with rocking at 4°C in 100 ml HBB.

    This blocking step (to reduce nonspecific binding) can also be performed overnight for convenience.

18. Incubate overnight with rocking at 4°C in BB containing 2.5–5 × 10^5 cpm/ml of radiolabeled fusion protein (from step 6).

    All eight filters can be placed in one 150-mm plate and probed with 30 to 40 ml of solution. The plate is wrapped in Parafilm and placed in a Plexiglas box for shielding. Alternatively, the filters can be placed with solution in a heat-sealable bag. The probe solution can be stored at 4°C and used for the subsequent secondary and tertiary screenings.

19. Wash membrane filters three times, 10 min each, in 100 ml BB with shaking at room temperature. Air dry and expose to film (see *UNIT 10.11*).

20. Using the large end of a Pasteur pipet, take an agarose plug at the position of the positive clone. Place into a 1.5-ml microcentrifuge tube and add 1 ml SM and 1 drop of chloroform.

    Agarose plugs can be stored at 4°C for months. Therefore, if many putative positive clones are identified in the primary screen, they can all be stored for future analysis if necessary.

21. Determine the titer by serial dilution and perform successive screening procedures to obtain purified clones.

    See Lech and Brent (1988) for serial dilution procedure and Quertermous (1987) for screening methods.

    Subsequent screens are performed on 100-mm LB plates with ~2000 pfu/plate for secondary screens and ~300 to 500 pfu/plate for tertiary screens. Before purified clones are obtained (usually during the tertiary screen), a control to eliminate clones that might interact with the GST fusion portion of the probe should be performed. To do this, cut the filters in half and probe one half with the labeled GST fusion to the protein of interest and the other with labeled GST alone or an unrelated control GST fusion protein (see Fig. 19.3.1).

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REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. All solutions should be prepared from sterile, autoclaved stock solutions, except Z′-KCl, which should be filter sterilized. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Binding buffer (BB)

20 mM HEPES·OH, pH 7.4
7.5 mM KCl
0.1 mM EDTA
2.5 mM $MgCl_2$
1% (w/v) nonfat dry milk

The solution can be prepared without milk and stored indefinitely at room temperature (add milk prior to use).

HEPES blocking buffer (HBB)

20 mM HEPES·OH, pH 7.4
5 mM $MgCl_2$
1 mM KCl
5% (w/v) nonfat dry milk

The solution can be prepared without milk and stored indefinitely at room temperature (add milk prior to use).

PKA buffer, 10×

200 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)
10 mM DTT
1 M NaCl
120 mM $MgCl_2$
Store up to 6 months at room temperature

Suspension medium (SM)

5.8 g NaCl
2 g $MgSO_4 \cdot 7H_2O$
50 ml 1 M Tris·Cl, pH 7.5 (*APPENDIX 2E*)
5 ml 2% (w/v) gelatin
H_2O to 1 liter
Sterilize by autoclaving.
Store up to several months at 4°C

Gelatin is prepared by adding 2 g gelatin to 100 ml H_2O, then autoclaving to dissolve when needed.

Tris-buffered saline with Triton X-100 (TBS-T)

10 mM Tris·Cl, pH 8.0 (*APPENDIX 2E*)
150 mM NaCl
0.05% (v/v) Triton X-100
Store up to 6 months at room temperature

Z′-KCl

25 mM HEPES·OH, pH 7.4
12.5 mM $MgCl_2$
20% (w/v) glycerol
100 mM KCl
1 mg/ml BSA
1 mM DTT
Filter sterilize
Store up to 6 months at 4°C

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Background Information

Historically, interacting proteins have been isolated by biochemical approaches, which required purification of the interacting protein for antibody production or microsequencing before a clone encoding the protein could be identified. Two of the molecular biological approaches described in this chapter, the yeast two-hybrid system (*UNIT 19.2*) and phage-based interaction expression cloning (this unit), however, directly yield a clone encoding the interacting protein.

Bacteriophage cDNA expression libraries are commonly screened using antibodies (see St. John, 1990 and references therein) or radiolabeled DNA probes to identify DNA-binding proteins (see Singh, 1991 and references therein). Modifications to identify interacting proteins include screening with the protein of interest (the bait protein) and detecting that protein with antibodies (Chapline et al., 1993). However, by screening with a radiolabeled protein probe, one avoids the additional incubations and washes which are necessary for immunodetection but that increase the likelihood of disrupting weak protein-protein interactions. ^{125}I-labeled protein probes have been used successfully to screen for interacting proteins (Hoeffler et al., 1991), although the technique described in this unit avoids the need to label the proteins with ^{125}I, and the complications of handling this isotope.

Protein probes autophosphorylated with ^{32}P were originally used to screen cDNA expression libraries in the isolation of proteins that interact with receptor protein kinases, a technique referred to as CORT (cloning of receptor targets; Skolnik et al., 1991; Lowenstein et al., 1992; Margolis et al., 1992). By introducing a PKA recognition site into the protein probe, the technique was made suitable for proteins that were not themselves protein kinases (Blanar and Rutter, 1992; Kaelin et al., 1992).

Phage-based interaction expression cloning as described in this unit has been used successfully to identify many interacting proteins, but may not be successful for all types of interactions. For example, many related proteins (known as A-kinase anchoring proteins, or AKAPs) have been identified by their ability to interact with the type II cAMP-dependent kinase regulatory subunit, RII (Carr and Scott, 1992). All the AKAPs can bind RII under denaturing conditions (Lester et al., 1996), illustrating that the success of interaction cloning is often dependent on the nature of the interactions and that interactions less dependent on three-dimensional structure may be favored. However, there are many examples of proteins identified by this technique that cannot interact under the denaturing conditions often used to confirm the binding (commonly referred to as overlay assays or Far Western analysis). For example, a protein domain identified by its interaction with a plant receptor–like protein kinase is capable of interacting with a denatured form of the bait protein, but is unable to interact when the interacting protein is denatured (Stone et al., 1994). This fact is consistent with the idea that immobilization on membranes denatures some proteins but not others.

Critical Parameters and Troubleshooting

The success of phage-based interaction expression cloning is inherently dependent on the quality of the protein probe used and the extent of representation of the bacteriophage cDNA library. GST fusion proteins are often obtained in high quantity in a soluble form, avoiding the necessity of solubilizing and refolding during protein purification from *E. coli* extracts. In most cases the PKA recognition site is readily accessible, and allows production of radiolabeled protein with a high specific activity.

The strength of the protein-protein interaction is also critical. Depending on the nature of the interaction, a filter-binding technique might not be suitable. This technique may not detect weak or transient interactions, such as an enzyme/substrate interaction, and techniques such as the yeast two-hybrid system (*UNIT 19.2*) might be more appropriate.

If no positive clones are identified or if background is high, it may be helpful to add reducing agents or detergents or to alter the salt conditions of the binding and wash solutions (Vinson et al., 1988). Moreover, denaturation and renaturation of the proteins on the filters using 6 M guanidine·HCl (Singh, 1991) may facilitate the recovery of clones expressing interacting proteins, because adsorption of the β-galactosidase fusion proteins to nitrocellulose filters may alter the conformation of the proteins.

For a discussion of common problems with this procedure and their diagnosis and possible solutions, see Table 19.3.1.

Anticipated Results

Depending on the nature of the interaction being sought by this technique, many interact-

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Table 19.3.1 General Troubleshooting Guide for Interaction Cloning

Problem	Possible cause	Solution
High background	Insufficient washing	Use more BB wash solution
	Poor choice of nitrocellulose membrane filters	Try a different supplier for membranes
	Insufficient blocking	Block overnight in HBB
No positive plaques	Poor-quality protein probe	Check the probe by SDS-PAGE and autoradiography
	Wash conditions too stringent	Reduce wash times
		Vary salt and/or detergent concentration
	Poorly folded library fusion proteins	Perform 6 M guanidine·HCl denaturation/renaturation (Singh, 1991)
Too many positive plaques	Wash and binding conditions insufficiently stringent	Increase salt and/or detergent in BB
	Library biased to proteins that interact with the affinity tag	Include unlabeled affinity tag in blocking, binding, and wash solutions

ing clones, none, or just a few may be identified. However, the technique is as simple as screening a library by hybridization and often well worth the effort. In any case, observed interactions should be confirmed by other means.

Time Considerations

The interaction cloning technique described in this unit is extremely rapid in comparison with other techniques to identify interacting proteins, such as the yeast two-hybrid system (*UNIT 19.2*). The technique does not require any unusual reagents that would not be readily available in any laboratory routinely engaged in molecular biology, other than the cAMP-dependent protein kinase. Once the appropriate recombinant fusion protein and cDNA expression library are obtained, the primary screen can be completed in a few days. Once the appropriate construct for expression of the GST-bait is obtained, purification of the recombinant GST-bait protein can be achieved in 1 day. ^{32}P labeling of the bait protein requires 2 hr (several additional hours if the optional SDS-PAGE and autoradiography are performed). The initial screening of the bacteriophage cDNA library takes 3 days; day 1 to prepare the filters and induce expression of library-encoded proteins, day 2 for blocking and probing overnight with ^{32}P-labeled bait protein, and day 3 for washing and autoradiography. Subsequent purification of the cDNA clones should only take an additional week. There are a number of steps that can be performed either rapidly or overnight, providing a great deal of convenience and flexibility.

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Key References

Blanar and Rutter, 1992. See above.

The basic protocol described in this unit is modified directly from the Blanar and Rutter protocol.

Huynh et al., 1985. See above.

Provides an excellent description of constructing and screening λgt11 cDNA expression libraries.

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SUPPLEMENT INDEX

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A new main index is provided once a year. Thereafter, with the next three supplements, a cumulative supplement index, which amends the main index, will be provided. Thus, readers should consult both the main index and the supplement index when seeking an entry, as page numbers may change for replacement units and new entries will added.

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Ch 2:1-2	Ch 2:1	Chapter 2 contents	Reflects revised *UNIT 2.5*
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COMPUTATIONAL ANALYSIS

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chromatography with on-line light scattering detectors are especially useful for characterizing proteins and glycoproteins (for review see Wen et al., 1996). *UNIT 7.8* provides an overview of the various light-scattering methods used to characterize proteins and macromolecular complexes. It should be noted that this method, like analytical centrifugation, provides direct molecular weight information without the use of calibration standards or models.

Circular dichroism and fluorescence spectroscopy are popular methods for determining protein conformation. Basic protocols for determining the circular dichroic spectra of proteins are described in *UNIT 7.6* and for determining the fluorescence spectra in *UNIT 7.7*. Circular dichroism and fluorescence spectroscopy are similar to most other spectroscopic methods in that careful interpretation of the data is required together with an understanding of the limitations and pitfalls of the method. These points are emphasized throughout these units.

Differential scanning calorimetry (DSC), described in *UNIT 7.9*, is an instrumental method used to measure heat energy uptake (heat capacity) in proteins undergoing thermal denaturation. This is accomplished by varying the temperature of a dissolved protein sample and measuring the difference in heat generated by the protein compared to a reference cell that contains solvent alone. This heat difference is related to the conformational energy of the protein molecule. In practice, when proteins undergo thermal denaturation, typically at 40° to 80°C, there is usually heat generated (i.e., it is an exothermic process); this heat can be very accurately measured by modern DSC instruments. The unit discusses the theoretical background of the method and details methods for actually performing DSC. For background on basic thermodynamic principles consult, for example, Tinco et al. (1995), and for overview of DSC applications in biotechnology see Chowdhry and Cole (1989). The Internet sites of the various instrument manufacturers are also useful sources of information.

Gel filtration is a technically simple method that is nonetheless one of the most powerful available for characterizing recombinant proteins. Working in an analytical mode using modern automated equipment, information on protein purity, conformation, and physical homogeneity can be obtained within a very short time. When the analysis is combined with mass spectroscopy, the gel filtration chromatogram can usually be fully interpreted to determine the chemical identity of all the peaks. *UNIT 7.10* details the complementary use of HPLC gel filtration and mass spectroscopy to analyze recombinant proteins.

CONFORMATIONAL ANALYSIS OF RECOMBINANT-PRODUCED PROTEINS

The assays for many protein functions, for example in the case of cytokines, can be time consuming and of relatively low precision. As proteins depend for their function on the correct 3D fold of the polypeptide chain, demonstration that the conformation of the recombinant protein is the same as that of the authentic protein should provide a more convenient means of characterizing the product. The methods described in *UNITS 7.4, 7.6 & 7.7*, although providing average structural information only, as compared with X-ray crystallography or NMR, are sensitive indicators of conformation and conformational change and, as such, can provide a highly specific "fingerprint" of a protein. Such conformational analysis will follow assurance of chemical authenticity and homogeneity, as described in *UNITS 7.1-7.3* (and elsewhere in the book). Urea gradient gel electrophoresis (*UNIT 7.4*) is a hydrodynamic technique that monitors the degree of compactness of the protein. It allows unfolding and refolding to be studied, leading to characterization of the protein in terms of its stability and kinetics. Near-UV circular dichroism (CD) results in spectra that are characteristic of the polar asymmetry of the environments of the aromatic

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residues, mainly tryptophan and tyrosine. Far-UV CD monitors the polypeptide backbone conformation, and hence, the secondary structure. Fluorescence spectra are determined chiefly by the polarity of the environment of the tryptophan and tyrosine residues and their interactions.The four techniques thus provide different, but complementary, fingerprints of the protein conformation that provide a high degree of assurance of nativeness within a few hours. For quality assurance of the native conformation and for general characterization, it is important to employ as broad a range of the available techniques as possible.

Besides confirming the nativeness of the folded conformation of a sample of a protein that has already been characterized, either as the previously produced recombinant protein or as the authentic protein, the techniques described here offer a rapid means of obtaining structural and dynamic information, together with estimates of the stability of the protein.

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Characterizing Recombinant Proteins Using HPLC Gel Filtration and Mass Spectrometry

It is essential to determine the identity, sequence integrity, purity, oligomeric state, and conformational integrity of a protein before structural or functional studies are carried out. This is true for proteins isolated from any source, but is particularly important for recombinant proteins, which are subject to several problems associated with their artificial overexpression in a heterologous host. These problems include expression in a denatured state, aggregation, mutations, incorrect translation (e.g., stop codon readthrough), incorrect proteolytic processing, and unwanted post-translational modifications. Often these problems cannot be detected using SDS-PAGE. Fortunately, most sources of heterogeneity in recombinant proteins can be identified by a preliminary analysis of the sample using a combination of analytical high-performance liquid chromatography/fast protein liquid chromatography (HPLC/FPLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Analytical gel filtration is used to detect and quantify contaminants in the sample (including protein aggregates), and can give an approximate measure of the protein size in solution (Stokes radius). Mass spectrometry is then used to determine the molecular weight of the protein to within a few daltons, which enables the detection and identification of incorrect sequence length and most chemical modifications of amino acid side chains. Apart from detecting unwanted modifications to the protein of interest, HPLC and mass spectrometry can be used to help characterize post-translational modifications such as glycosylation (see Chapter 12).

In this unit, analytical HPLC gel filtration is used to characterize a purified recombinant protein sample in the Basic Protocol. A method for determining the Stokes radius of a protein using HPLC is described in Support Protocol 1. MALDI-MS is used to characterize a protein sample in Support Protocol 2.

NOTE: Milli-Q-purified or equivalent quality water should be used for all buffers.

STRATEGIC PLANNING

Selecting Column Type and Size

The first step in planning a size-exclusion fractionation is selecting the type of column to be used. Several factors need to be considered. Most importantly, the molecular weight of the recombinant proteins to be analyzed should fall within the fractionation range of the column, which is determined by the pore size of the particles. The fractionation range should be large enough to encompass the molecular weights of all species to be separated, and the protein of interest should be near the midpoint of the fractionation range. The fractionation range may be increased by placing two columns with different pore sizes in series.

Further selection of the column depends upon several related factors: the required resolution, the sample volume, and the desired run time. For analytical gel filtration, the aim is to maximize resolution while minimizing run time. Resolution is affected by the particle size and size distribution, as well as the column length and the flow rate (for a detailed discussion, see *UNITS 8.1 & 8.3*, or Neue, 1997). Columns with small, rigid, well-defined particles (<15 µm diameter) show the best resolution while allowing fast flow rates and therefore short run times (12 to 60 min). Table 7.10.1 lists several common commercial prepacked columns that feature small particle sizes and good resolution. The disadvantage of these columns is that the small particle sizes lead to high back pressures, requiring an isocratic chromatography system that can operate at moderate pressure (FPLC; 200 to 600 psi) or preferably higher pressure (HPLC; >500 psi). Resolution may

Contributed by Gillian E. Begg, Sandra L. Harper, and David W. Speicher
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Table 7.10.1 Common Prepacked Columns for HPLC/FPLC Size-Exclusion Chromatography[a]

Column type	Fractionation range (kDa)	Resin[b]	Particle size (μm)	Dimensions available (i.d. × length, mm)	Supplier[c]
Microanalytical					
TSK Super SW2000	5-150	S	4	4.6 × 300	TH
TSK Super SW3000	10-500	S	4	4.6 × 300	TH
Analytical					
TSK G2000SW$_{XL}$	5-150	S	5	7.8 × 300	TH
TSK G3000SW$_{XL}$	10-500	S	5	7.8 × 300	TH
TSK G4000SW$_{XL}$	20-10,000	S	8	7.8 × 300	TH
TSK G2000SW	5-100	S	10	7.5 × 300 7.5 × 600	TH
TSK G3000SW	10-500	S	10	7.5 × 300 7.5 × 600	TH
TSK G4000SW	20-7000	S	13	7.5 × 300 7.5 × 600	TH
Bio-Sil SEC 125	5-100	S	5	7.8 × 300	BR
Bio-Sil SEC 250	10-300	S	5	7.8 × 300	BR
Bio-Sil SEC 400	20-1000	S	5	7.8 × 300	BR
Protein-Pak 60	1-20	S	10	7.8 × 300	WA
Protein-Pak 125	2-80	S	10	7.8 × 300	WA
Protein-Pak 200SW	1-60	S	10	8.0 × 300	WA
Protein-Pak 300SW	10-300	S	10	8.0 × 300	WA
Superose 12 HR 10/30	1-3000	CL A	10	10.0 × 300	APB
Superdex Peptide	0.1-7	CL A/D	13	10.0 × 300	APB
Superdex 75 HR	3-70	CL A/D	13	10.0 × 300	APB
Superdex 200 HR	10-600	CL A/D	13	10.0 × 300	APB
Superose 6 HR	5-5000	CL A	13	10.0 × 300	APB
Preparative					
TSK G2000SW	5-100	S	13	21.5 × 300 21.5 × 600	TH
TSK G3000SW	10-500	S	13	21.5 × 300 21.5 × 600	TH
TSK G4000SW	20-7000	S	17	21.5 × 300 21.5 × 600	TH
TSK G2000SW	5-100	S	20	55.0 × 300 55.0 × 600	TH
TSK G3000SW	10-500	S	20	55.0 × 300 55.0 × 600	TH
Superdex 30 HiLoad	1-10	CL A/D	34	16.0 × 600 26.0 × 600	TH
Superdex 75 HiLoad	3-70	CL A/D	34	16.0 × 600 26.0 × 600	TH
Superdex 200 HiLoad	10-600	CL A/D	34	16.0 × 600 26.0 × 600	TH

[a]Data reported by manufacturers.

[b]Abbreviations: CL A, cross-linked agarose; CL A/D, cross-linked agarose/dextran; S, coated silica.

[c]Abbreviations: APB, Amersham Pharmacia Biotech; BR, Bio-Rad; TH, TosoHaas; WA, Waters. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

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be further enhanced by increasing the length of the column; if a longer column is not available, two columns may be placed in series to achieve the same effect. Low-pressure gel filtration columns with softer, larger particles are generally less expensive and allow larger protein loads, but require lower flow rates, which markedly increases the run time for the separation while providing reduced resolution even at low flow rates.

If it is necessary to scale up an analytical separation, the sample volume will determine the size of the column required—the larger the sample volume and amount of protein to be separated, the greater the column volume needed. One option is to increase the length of the column; for example, a 60-cm column has twice the sample volume capacity of a 30-cm column, with little loss of resolution. Increasing the diameter of the column will also increase the maximum load in proportion to the square of the increase in diameter. Table 7.10.1 lists prepacked columns for both preparative work (i.d. = 21.5 or 55 mm) and analytical work (i.d. ≤ 10 mm).

Another factor to consider when selecting a column is the potential for interactions between the sample and the column. For example, silica-based columns are available in the smallest particle sizes and can be operated at higher pressures compared to agarose gels; however, some adsorption can occur due to electrostatic interactions with unmodified silanol groups. Usually these effects can be minimized by maintaining at least a physiological ionic strength. Stainless steel columns can withstand high back pressures, but also have drawbacks. For example, some samples might interact with the metal surfaces (especially stainless steel end frits) and common buffer components (e.g., halogens such as chloride) can react with incompletely passivated stainless steel surfaces such as the compression screws. For samples or buffer systems that are incompatible with stainless steel, glass columns should be used.

Selecting Instrument Type

An isocratic (one pump, one buffer) HPLC or FPLC system is required for the columns described in this unit. Selection of an appropriate chromatography system involves two major decisions: operating pressure range and whether or not biocompatibility is needed. FPLC, HPLC, and biocompatible systems are appropriate for different types of applications. An FPLC system is used to run columns that require moderate back pressure (typically 50 to 600 psi). An HPLC system is used to run columns with higher back pressures (typically >500 psi). Biocompatible systems are available for operation in a variety of pressure ranges and are used in cases where the protein of interest may be adversely affected by trace metal ions that could exist with a system containing stainless steel. Biocompatibility is a heterogeneously applied term, generally assumed to mean a system with few or no stainless steel components. The importance of biocompatibility for most protein applications has not been clearly established.

Many companies sell integrated computer-controlled systems that consist of reliable pumps, an injector, a UV detector, a fraction collector, and a computer output for data collection and manipulation. Of course, a dual-pump HPLC or FPLC system may be used by utilizing only one of the pumps. It is most critical to have a pumping system that can deliver a constant flow rate in the desired range, which is typically 0.1 to 1.5 ml/min for analytical columns and up to 5 ml/min for preparative columns. Because of the small number of samples typically analyzed, an autosampler is not necessary. However, it is important to have a computer integrated with the injector to ensure precise starting points for sample injection. While most binary HPLC and FPLC systems are relatively expensive, there are less expensive alternatives, such as integrated isocratic systems that cover the required pressure range (e.g., 0 to 2500 psi) and cost under $10,000 (e.g., D-Star Instruments Integrated Isocratic HPLC system, available through Thomas Scientific).

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Selecting Appropriate Buffers

The buffer selected for gel filtration should be optimized for protein stability. Highly hydrophobic or membrane proteins, for example, require a nondenaturing detergent in the buffer (see next section). Silica-based resins are not stable above pH 7.5, and stainless steel columns should typically not be used with halogens. Buffer type and strength may affect resolution and mass recovery. The ionic strength of the buffer is also important. In high–ionic strength buffers (>1 M), hydrophobic interactions between the protein and the resin may occur, while at low ionic strength (<0.1 M), ionic interactions may occur.

Analysis of Membrane Proteins in Detergents

The separation of membrane proteins in the presence of detergents (typically a nonionic or zwitterionic detergent) is the same in principle as separation in aqueous solutions. However, in the presence of detergent, the apparent size of the protein will probably increase due to detergent binding to the protein. For this reason, the fractionation range of gel filtration columns specified for water-soluble proteins will not apply to detergent-solubilized proteins (Schagger, 1994). Accurate size determination is not feasible in the presence of detergents, since membrane proteins will bind detergent molecules and/or interact with detergent micelles to differing degrees.

The procedure for gel filtration in the presence of detergent is essentially as described in the Basic Protocol, with a few additional factors to take into account. First, the protein sample should be equilibrated with the detergent prior to the run. Detergents with a low critical micelle concentration (CMC) do not dialyze well, so the detergent can be added directly to the sample, or a desalting column can be used for buffer exchange. The choice of detergent is another important consideration. If the protein elution profile is to be detected via UV absorbance, for example, the detergent needs to be transparent at the wavelength selected (for example, detergents containing aromatic ring systems, e.g., Triton, absorb strongly at 280 nm), unless the concentration of detergent can be kept low enough to keep the absorbance signal within the linear range of the UV detector. However, the gel filtration buffer should contain detergent at a concentration well above the CMC (i.e., ≥4×) to prevent association of membrane proteins (Schagger, 1994). The micelle size is also important, as large micelles will change the elution behavior of proteins more than small micelles, and may be harder to separate from the protein of interest.

A common problem in gel filtration of membrane proteins is adsorption of protein to the resin or the column. The extent of this problem depends upon the detergent type and concentration, as well as the resin type and column hardware. Unfortunately, predicting the column and detergent that will minimize adsorptive losses is difficult, and a trial-and-error approach may be needed to find the optimum combination.

BASIC PROTOCOL

HPLC ANALYSIS OF RECOMBINANT PROTEINS

This protocol describes analytical HPLC gel filtration of soluble recombinant proteins (refer to *UNITS 8.1 & 8.3* for a complete description of gel filtration chromatography). Analytical HPLC is used to evaluate sample heterogeneity and quantify sample components. For example, HPLC may be used to monitor the proteolytic cleavage of a fusion protein (Fig. 7.10.1), to separate two or more oligomers, or to determine the amount of aggregate or other contaminates in a sample. Although the protocol is written for HPLC systems, FPLC and HPLC are largely interchangeable (see Strategic Planning), and either can be used.

As analytical HPLC is often used to compare different preparations of a recombinant protein, to monitor the homogeneity of a sample over time, or to compare a recombinant

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protein with standards, accurate and reproducible determination of retention time is critical. For this reason it is important to use high-precision chromatography pumps that provide a constant, accurate flow rate. It is also important that the column packing be in good condition and maintained at a constant temperature; typically 4°C is used to minimize potential proteolysis of the protein. This can be accomplished by placing either the column or the entire chromatography system in a cold cabinet.

Materials

Gel filtration buffer (see recipe)
Protein standard (e.g., Bio-Rad gel filtration standard)
Protein sample
0.05% (w/v) sodium azide or 20% (v/v) ethanol

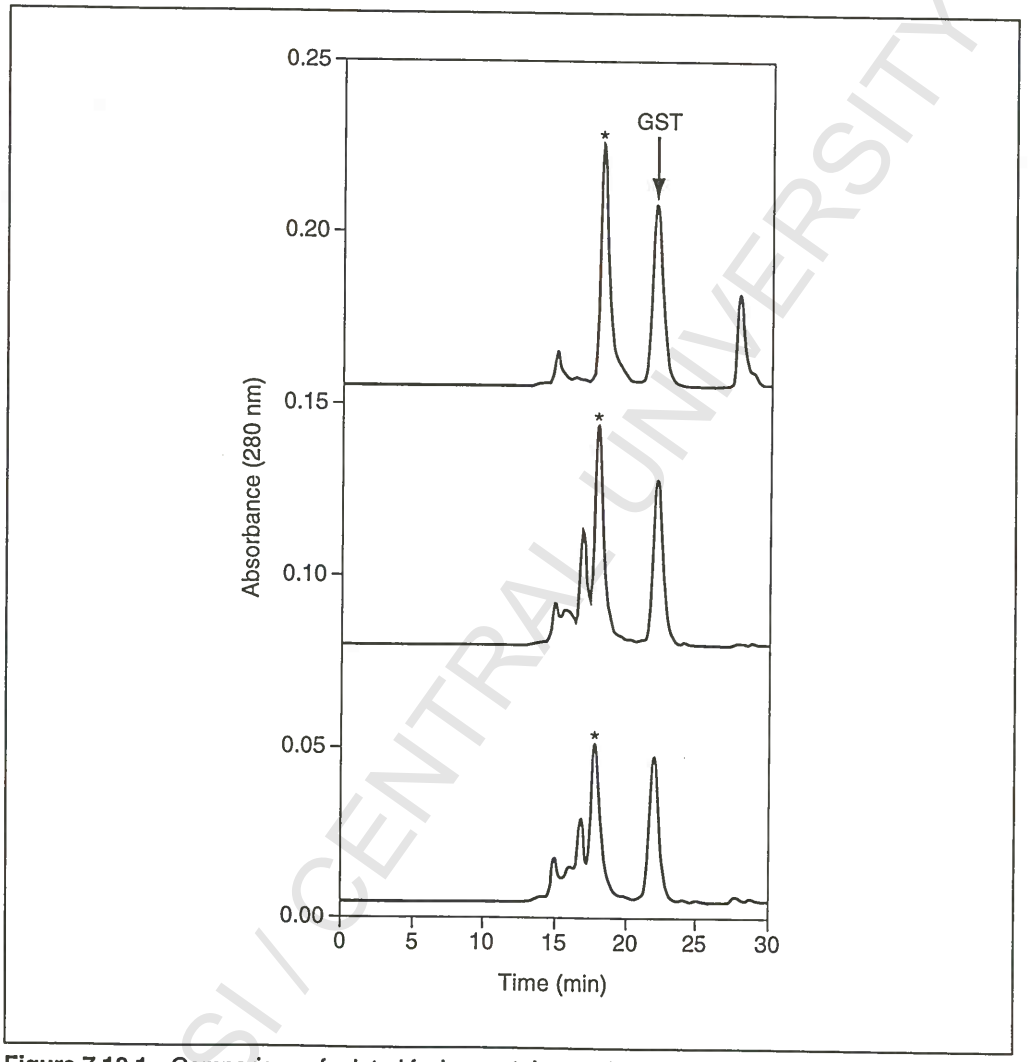

Figure 7.10.1 Comparison of related fusion proteins analyzed immediately after protease cleavage using analytical HPLC gel filtration to evaluate potential self-aggregation. The top panel shows 100 µg of the wild-type 52-kDa recombinant protein, and the lower panels show two different mutants. The asterisk indicates the retention time of the monomeric proteins. The elution position of the glutathione-S-transferase (GST) moiety is also indicated. Peaks eluting earlier than the monomeric form of the protein are self-aggregates. These results show that the mutants, but not the wild type, rapidly aggregate during protease digestion. When monomeric forms of the mutants were isolated by preparative HPLC gel filtration and reanalyzed by analytical gel filtration, a substantial increase in aggregation occurred in a concentration- and time-dependent manner (data not shown).

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Isocratic liquid chromatography system (HPLC or FPLC)
Prepacked HPLC or FPLC column (Table 7.10.1)
0.2-μm filters

1. Select an appropriate column for the type of proteins to be analyzed. Refer to Strategic Planning and Table 7.10.1 for a listing of prepacked analytical HPLC columns.
2. Prepare an isocratic liquid chromatography system as in Basic Protocol 1 of *UNIT 8.3*, steps 14 to 16 (also see Strategic Planning of this unit).

   It is desirable to have an HPLC UV detector in line with outputs to both a strip chart recorder and to a computer for peak quantification.
3. Filter gel filtration buffer that is compatible with both the column and the sample, using a 0.2-μm filter to degas and remove any small particles that may clog the column frit.

   Refer to the manufacturer's recommendations for column-compatible buffers. The column buffer does not need to be identical to the buffer of the injected sample, as gel filtration can also be used to exchange buffers; however, the sample should be tested with the column buffer to ensure that no precipitate occurs when the sample enters the column.

   If bacteriostatic agents are not used, buffer should be filtered daily.
4. Equilibrate the column with three column volumes of gel filtration buffer.

   Do not exceed the manufacturer's recommended flow rates or pressure limits. If operating at low temperatures, it may be necessary to decrease the flow rate as the viscosity of the buffer may increase, generating higher back pressures.
5. Pass a protein standard through a protein-compatible 0.2-μm filter or centrifuge at 10,000 × *g* for 10 min to remove particulate matter.
6. Rinse the sample application loop with gel filtration buffer and evaluate the performance of the column by injecting the protein standard.

   Figure 7.10.2 shows chromatograms obtained with columns showing optimal (upper panel) and poor performance (lower panel). Note the reduced peak heights, increased peak widths, and decreased separation between peaks in the lower chromatogram.
7. Pass a protein sample through a 0.2-μm filter or centrifuge at 10,000 × *g* for 10 min to remove particles.
8. Rinse the sample application loop again and inject an aliquot of the recombinant protein to be analyzed.

   The lower limit of protein concentration is determined by the sensitivity of the detector used, while the upper limit is influenced by multiple factors including sample viscosity, potential nonideal behavior of the sample at high concentrations (e.g., aggregation), and degradation of resolution between adjacent peaks as protein loads increase. For optimum resolution, sample volumes should be ≤1% of the total column volume (column volume [ml] = $\pi r^2 l$, where radius [r] and length [l] are in cm). For initial analytical separations, a sample volume of ~0.5% of the column volume can generally be used, with a total protein concentration of ~0.5 to 1.0 mg/ml.

   Figure 7.10.3 shows a typical analysis of a 55-kDa recombinant protein by SDS-PAGE, HPLC, and MALDI-MS.
9. *Optional:* Collect fractions of the eluting proteins.

   Collection of fractions during a gel filtration separation allows subsequent identification of individual peaks with other methods such as MALDI-MS (see Support Protocol 2 and Fig. 7.10.5C), SDS-PAGE (UNIT 10.1 and Fig. 7.10.5B), immunoblotting (UNIT 10.10), or N-terminal sequencing (UNIT 11.10). However, it should be noted that because the amount of protein typically loaded on an analytical HPLC column is small, and proteins are diluted

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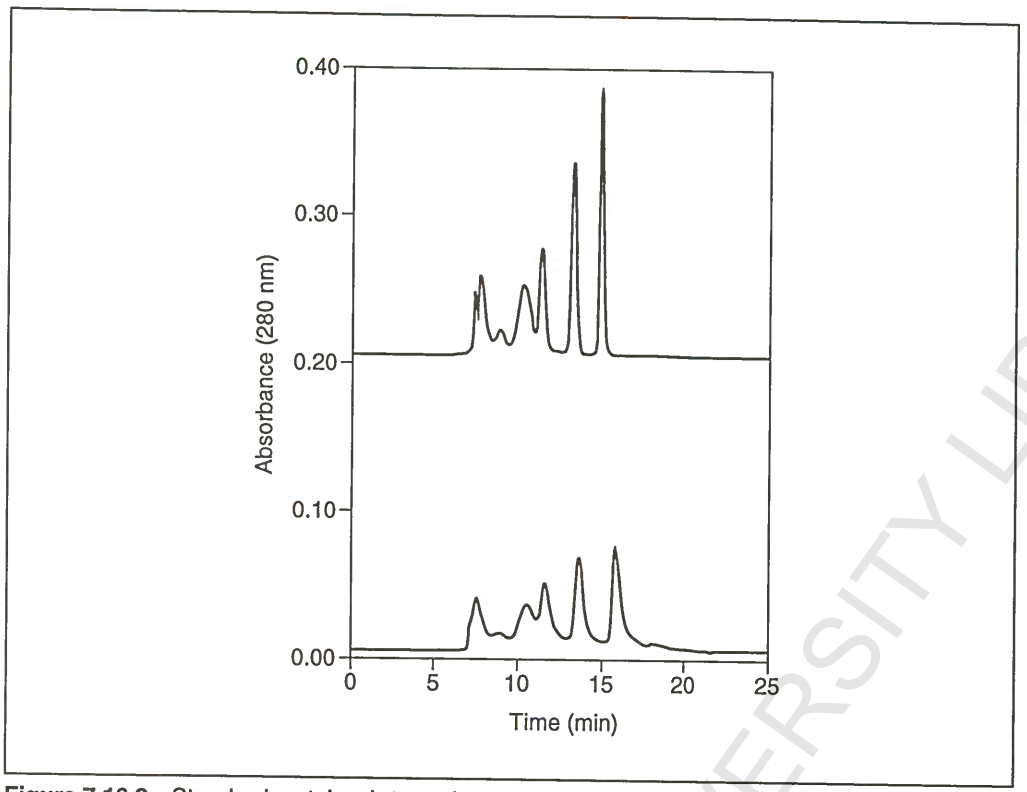

Figure 7.10.2 Standard protein mix to evaluate column performance. A comparison between new (upper panel) and old (lower panel) TSK G3000SW$_{XL}$ (TosoHaas) columns. In this case, the decreased resolution of the old column was primarily attributed to a void that had formed at the top of the column (see Troubleshooting).

during the separation, the protein yield and/or concentration may not be high enough for some forms of analysis. In some cases the fractions may be pooled and/or concentrated prior to analysis, otherwise the separation may have to be scaled up (see Strategic Planning). If collecting fractions, it is a good idea to collect through the entire run time of the separation, using fraction volumes that will provide at least four to six fractions across a typical peak (this can be estimated from the average peak widths of standard proteins).

10. Store column in a solution containing a bacteriostat such as 0.05% sodium azide or 20% ethanol. Refer to the manufacturer's recommendations regarding column storage.

DETERMINATION OF STOKES RADIUS BY HPLC GEL FILTRATION

SUPPORT PROTOCOL 1

This protocol describes the use of HPLC gel filtration to rapidly determine the Stokes radius of proteins (also see *UNIT 8.3*, Basic Protocol 3). Protein standards may be ordered individually or as part of a kit. Table 8.3.8 provides a list of commercially available standards. It is important to note that a Stokes radius is a measure of molecular size and not molecular mass; effectively, it is the radius of an equivalent hydrated sphere (Cantor and Schimmel, 1980). Thus, an approximate molecular mass may be obtained only when the protein of interest and the protein standards that are used in calibration have the same shape. As most standards are roughly spherical, the protein of interest should also be spherical. A better estimate of molecular mass (independent of shape) may be calculated from the Stokes radius combined with a sedimentation coefficient, which can be obtained using analytical ultracentrifugation or sucrose gradients (Seigel and Monty, 1966; *UNIT 7.5*).

1. Select a column of appropriate fractionation range for samples of interest and equilibrate the column in a compatible gel filtration buffer (see Basic Protocol, steps 1 to 4).

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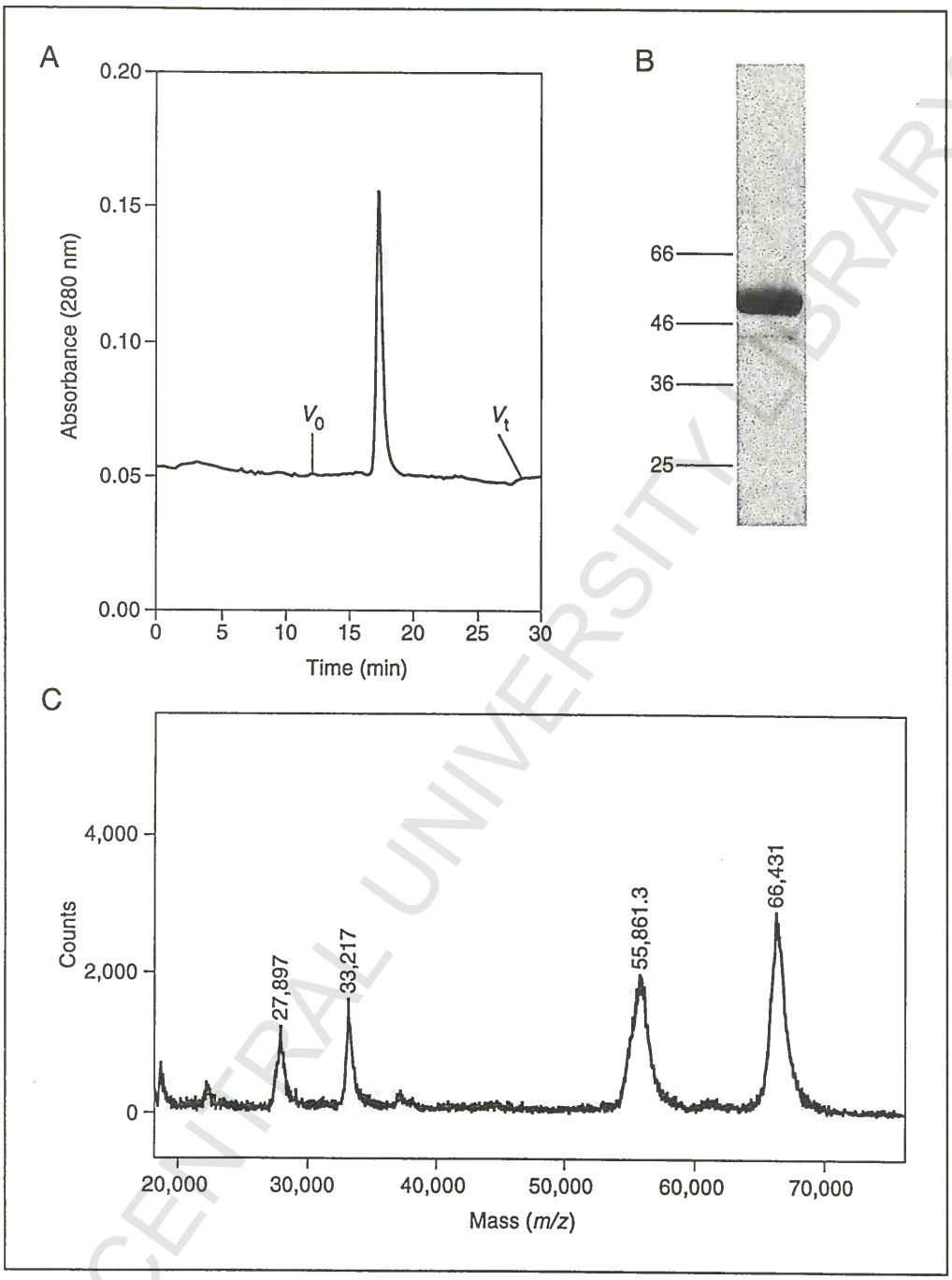

Figure 7.10.3 Analysis of a purified recombinant protein by HPLC gel filtration, SDS-PAGE, and mass spectrometry. (**A**) HPLC chromatograph of an α-actinin recombinant (25 µg injected). (**B**) SDS-PAGE Coomassie blue–stained gel showing the α-actinin recombinant. (**C**) Mass spectra of α-actinin recombinant protein. The apparent mass from SDS-PAGE is close to the expected mass. The HPLC chromatograph shows a single species, which migrates near the midpoint of the fractionation range, indicated by the void volume (V_0) and the total column volume (V_t). However, when mass analysis was performed, the actual mass observed ($[M + H]^+$ = 55,861.3 or [M] = 55,860.3) was 738 Da larger then the sequence molecular mass, corresponding to a readthrough of the insert stop codon. Instead, the sequence terminated at the in-frame stop codon in the cloning vector. The peak at 66,431 is the single protonated form of BSA, an internal standard. The peaks at 27,897 and 33,217 correspond to the double-protonated species of the recombinant and standard, respectively.

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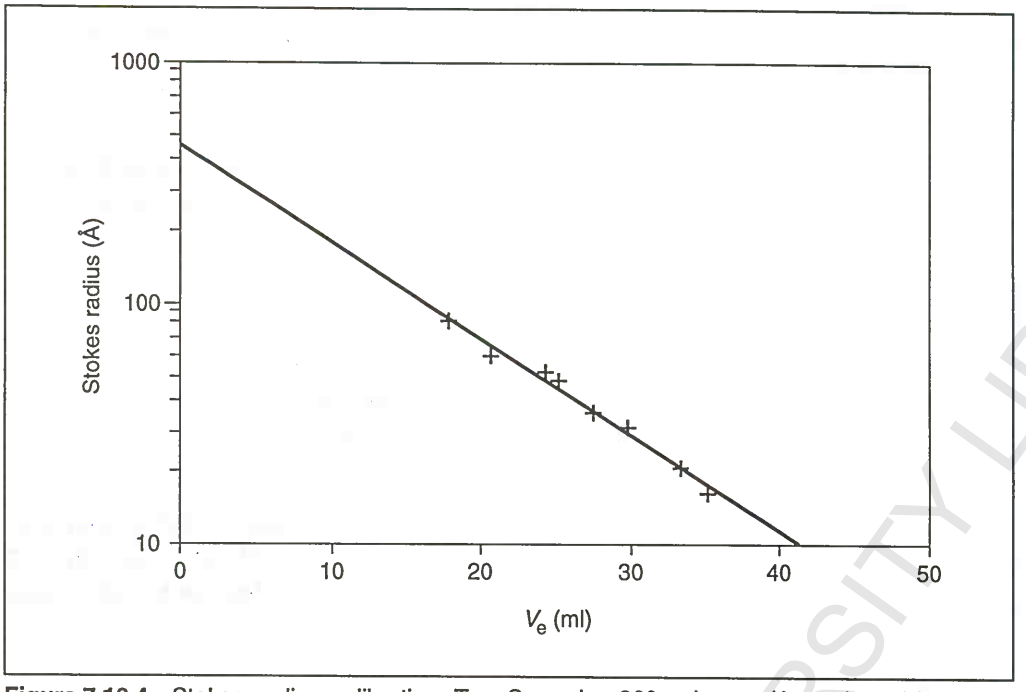

Figure 7.10.4 Stokes radius calibration. Two Superdex 200 columns (Amersham Pharmacia Biotech) were connected in series and run at a flow rate of 0.5 ml/min in PBS, pH 7.4, containing EDTA, phenylmethylsulfonyl fluoride, and sodium azide. Individual protein standards from the Amersham Pharmacia Biotech high- and low-molecular-weight gel filtration kits were prepared at a concentration of 1 to 5 mg/ml in gel filtration buffer. A total of 50 µg of each standard was injected. Samples included: ribonuclease A (16.4 Å), chymotrypsinogen A (20.9 Å), ovalbumin (30.5 Å), albumin (35.5 Å), aldolase (48.1 Å), catalase (52.2 Å), ferritin (61.0 Å), and thyroglobulin (85.0 Å). The retention time was multiplied by the flow rate (measured as 0.45 ml/min) to determine the elution volume (V_e), which was plotted verses the log of the known Stokes radii (R_s). The equation for the fitted line is $\log R_s = -0.04 V_e + 2.65$. This equation can then be used to determine the Stokes radius of an unknown, by injecting a small amount of the sample, determining V_e, and solving the equation for R_s.

2. Dissolve individual protein standards to a concentration of 1 to 5 mg/ml in gel filtration buffer. Pass through a protein-compatible 0.2-µm filter or centrifuge at 10,000 × *g* for 10 min to remove particulate matter.

   Protein standards can be mixed together to save time and reduce the number of injections; however, some proteins may interact and affect the elution of other proteins in the mix.

3. Inject each protein standard or the protein standard mixture onto the column. Rinse the sample application loop with buffer prior to each injection.

   For accurate comparison of the standard and sample retention times, the volume of the protein standard injected onto the column should be the same as that of the sample to be tested.

4. Determine the elution volume (V_e) of each standard by multiplying the retention time by the flow rate.

   Because pump performance will vary on most systems as piston seals wear, it is a good idea to check the actual flow rate by measuring the precise time required for 10 ml of eluant to be collected in a 10-ml graduated cylinder or volumetric flask.

5. Construct a graph plotting the log of the known Stokes radius (R_s) versus elution volume for each standard.

   Figure 7.10.4 shows a graph of $\log R_s$ versus V_e for a series of protein standards run on a Superdex 200 column.

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6. Fit the points with a straight line. Determine the slope (a) and y offset (b) of the line.

   Most graphing or spreadsheet programs may be used to perform linear regression analysis on the data points, giving the slope and offset of the fitted line.

7. Inject protein samples with unknown Stokes radii onto the same column. Calculate V_e and use the equation $\log R_s = aV_e + b$ to determine the Stokes radius of each unknown (see Fig. 7.10.4).

SUPPORT PROTOCOL 2

MALDI-MS ANALYSIS OF RECOMBINANT PROTEINS

The following protocol describes a basic method for MALDI-MS analysis of proteins carried out on a Voyager RP spectrometer (PerSeptive BioSystems). See *UNITS 16.1-16.3* or Beavis and Chait (1996) for a more detailed discussion of MALDI-MS theory and methods.

Two matrices are commonly used for recombinant proteins: α-cyano-4-hydroxycinnamic acid (for proteins <20 kDa) and sinapinic acid (for proteins >20 kDa), each in 33% acetonitrile/0.1% trifluoroacetic acid. The optimum matrix may have to be determined empirically for each sample.

Materials

1 to 10 pmol/µl HPLC-purified protein sample of interest (see Basic Protocol)
MALDI-MS-compatible buffer (e.g., 10 mM ammonium bicarbonate, pH 8)
Molecular weight standards
Saturated matrix solution (see recipe)
Sample target (e.g., gold or stainless steel plate)
Matrix-assisted laser desorption/ionization (MALDI) mass spectrometer (Voyager RP, PerSeptive BioSystems)

1. *Optional:* Dialyze an HPLC-purified protein sample into a MALDI-MS-compatible buffer.

   This step removes buffer components that may interfere with ionization or crystal formation. See Table 16.2.1 for tolerance limits of buffer components. Sodium azide, which is typically used in HPLC gel filtration buffers as a bacteriostatic reagent, is particularly problematic if not removed by dialysis, as it causes strong signal suppression.

   Very small samples (<300 µl) can be dialyzed in a microcentrifuge tube (Overall, 1987).

2. Dilute sample to a concentration of 1 to 5 pmol/µl with water or MALDI-MS buffer.

3. Select and prepare molecular weight standards.

   Two mass peaks are typically used to calibrate the mass spectrometer; these can be the singly and doubly charged species of a single standard, such as protein A, or two different protein standards. Ideally, the molecular weight of the standards should bracket the expected masses of the sample. See Table 7.10.2 for a list of typical molecular weight standards. The most accurate calibration is obtained when the standards are included in the sample of interest (internal calibration). However, the standard may suppress ionization of the recombinant protein, in which case the ratios of standards and sample must be adjusted empirically or an external calibration must be used.

4. Apply 0.5 µl saturated matrix solution to a sample target and allow to air dry. Apply 0.5 µl protein sample onto the dried matrix and allow to dry.

   Alternatively, mix 2 µl matrix with 2 µl sample in a microcentrifuge tube, apply 1 µl to the sample target, and allow to air dry.

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Table 7.10.2 Common Molecular Weight Standards for MALDI-MS of Proteins

Standard	Molecular weight (Da)	Concentration (pmol/μl)	Matrix
Bradykinin	1061.2	1	HCCA[a]
Insulin B	3496.9	1	HCCA
Insulin	5734.6	2-4	HCCA
Ubiquitin	8565.9	1-2	HCCA
Cytochrome *c*	12231.9	1-2	HCCA
α-Lactalbumin	14071.1	1-2	HCCA
Trypsin inhibitor A	20091.7	1-2	Either
α-Chymotrypsin	25234.7	1-2	Either
Ovalbumin	44401.0	3-5	Sinapinic acid
Protein A	44614.0	3-5	Sinapinic acid
BSA	66341.0	3-5	Sinapinic acid

[a]HCCA, α-cyano-4-hydroxycinnamic acid.

5. Insert sample target into a MALDI mass spectrometer and follow the manufacturer's procedure for obtaining a spectrum.
6. Compare the mass obtained with that expected from the amino acid sequence of the recombinant protein.

The masses should agree to within ~0.1% when an external standard is used, and ~0.03% using an internal standard. If this is not the case, or if additional peaks are present, there may be some modification of the protein. Table 7.10.3 lists some common modifications seen with recombinant proteins. Figure 7.10.5 shows typical MALDI-MS results.

Table 7.10.3 Interpreting Mass Spectra of Recombinant Proteins

Problem/change	Comment	Typical mass change
Stop codon readthrough (Fig. 7.10.3)	Stop codon in the construct is missed; translation continues until the next stop codon, often in the vector	+50 to 1000
Disulfide adduct	Disulfide bond formed between a reducing agent and a highly reactive cysteine in the recombinant protein	+76 (2-mercaptoethanol) +119 (dithiothreitol) +305 (glutathione)
Disulfide dimer	Formed between two reactive cysteines	2× expected mass
Protease degradation	For example, due to contaminating proteases, or secondary cleavage sites for proteases used to cleave fusion protein	Variable size reduction
Incomplete removal of tag sequences	For example, His tag (~6 His) or Flag sequence	Increased mass should equal calculated tag sequence
Post-translational modifications (e.g., glycosylation; Fig. 7.10.5C)	Only in eukaryotic expression systems; glycosylation usually gives broad or multiple peaks	Variable (+ ≥300)
Removal of initiation Met residue		−131

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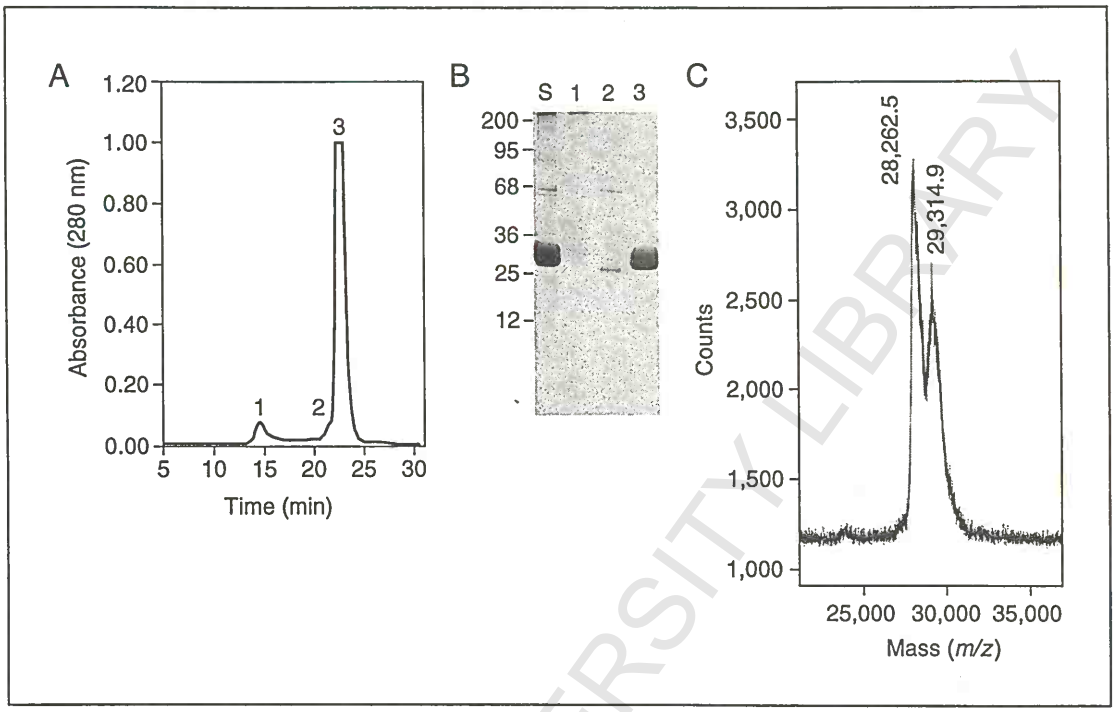

Figure 7.10.5 Identifying post-translational modifications using MALDI-MS. (**A**) HPLC chromatograph of a recombinant extracellular domain expressed in *Sf*9 insect cells. The recombinant protein (peak 3) is effectively separated from high-molecular-weight contaminants (peaks 1 and 2). (**B**) SDS-PAGE of the sample before gel filtration (lane S), and fractions taken from HPLC peaks 1 to 3 (lanes 1 to 3). (**C**) MALDI mass spectrum of the purified extracellular domain from peak 3. Two broad peaks are detected, with molecular masses 0.6 to 1.7 kDa greater than the molecular mass calculated from the sequence (27,531 Da), indicating heterogeneous glycosylation of the protein. The sequence mass was calculated after determining the site of signal peptide cleavage using N-terminal sequence analysis of the intact recombinant.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Gel filtration buffer

PBS (*APPENDIX 2E*) or other buffer compatible with both the column and sample. Protease inhibitors (e.g., 1 mM EDTA, 0.15 mM phenylmethylsulfonyl fluoride) as well as a bacteriostat (0.05 % sodium azide) may also be added to the buffer. A reducing agent (2-mercaptoethanol, dithiothreitol, or tris(2-carboxyethyl)phosphine hydrochloride [TCEP·Cl]) may also be included to prevent proteins containing reactive cysteines from forming disulfide linkages.

Buffers should be degassed and made fresh each week unless antimicrobial agents have been added, in which case it may be used for up to one month.

Saturated matrix solution

Prepare 10 mg/ml matrix (α-cyano-4-hydroxycinnamic acid or sinapinic acid) in 33% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid. Centrifuge briefly to sediment undissolved matrix. Use the saturated solution to prepare samples. Stored for up to 2 weeks at 4°C, protected from light.

For optimum sensitivity, the α-cyano-4-hydroxycinnamic acid matrix supplied by the manufacturer can be repurified (see UNIT 16.3, Support Protocol).

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Background Information

Analytical HPLC gel filtration and MALDI-MS are relatively rapid and convenient methods that can be used to detect (and in some cases help identify) many protein modifications and potential self-aggregation seen after expression of recombinant proteins. Gel filtration chromatography is one of the oldest methods used to characterize proteins, and its resolution and speed are substantially improved by modern small-particle high-performance resins. The general aim of analytical HPLC gel filtration is to separate and quantify the components of a protein mixture based on their solution size. In the case of recombinant proteins, the method is used primarily to quantify contaminants, especially self-aggregates (Fig. 7.10.1), and to obtain an approximate Stokes radius of each of the sample components. One of the major advantages of gel filtration over other techniques (e.g., SDS-PAGE) is that it is nondenaturing, enabling the separation, quantification, and approximate size determination of oligomers and aggregates of the protein of interest as they occur in solution. Furthermore, gel filtration can be performed in a variety of solution conditions, so the effects of ionic strength, pH, temperature, or reducing agents on the sample may be tested. Other advantages of the technique are that the sample is recoverable—allowing further analyses (e.g., SDS-PAGE or mass spectrometry) to be performed on the separated components—and that the analysis time is relatively short (typically ~30 to 60 min).

There are some limitations of gel filtration, and additional techniques should be used to fully characterize recombinant proteins. Depending on the sensitivity and detector wavelength used, HPLC analysis may fail to detect some contaminants that might be seen by SDS-PAGE, such as proteins without tyrosine or tryptophan residues, which will not have substantial absorbance at 280 nm. For size determination, HPLC gel filtration is fast, requires very little protein, and gives excellent fractionation, but has inherent drawbacks, such as the potential for retardation of protein elution caused by electrostatic or hydrophobic interactions with the column matrix. For comparison, velocity sedimentation (*UNIT 7.5*) requires no calibration standards and there is no solid phase to interact with the protein, but the fractionation of complex mixtures is not as great. It is important to note that, due to the large variations in protein shape and degree of hydration, a Stokes radius is often not a good indicator of oligomeric state. While HPLC is a rapid first analysis method, the oligomeric state of a protein should be definitively determined using equilibrium sedimentation in an analytical ultracentrifuge (*UNIT 7.5*).

MALDI-MS is an extremely valuable tool for the protein chemist, allowing determination of protein molecular mass within a few daltons, regardless of the size, shape, or hydration of the protein. Other advantages of this technique are that it can be applied to protein mixtures, only ~10 pmol of protein is needed, and the analysis can be completed in a few minutes. The drawbacks of this technique are that some proteins are difficult to ionize and that it is not quantitative, as different proteins can yield highly variable signal intensities. Thus, the utility of MALDI-MS is the accurate mass determination of the protein of interest, which can detect most post-translational and artifactual modifications of that protein. It should also be noted that while obtaining the mass predicted by the sequence of the protein of interest is a good indication that the sequence is correct, confirmation of sequence identity may also require N-terminal sequence analysis of the intact protein and HPLC/MS mapping of tryptic peptides (*UNIT 7.3*).

Critical Parameters

The maximum sample volume that is applied to the column should not exceed 0.5% to 1% of the total column volume for optimal resolution of the sample components. Sample capacity may be increased by using a larger-diameter column. Another way of achieving maximum resolution of components is to place two or more columns in series, effectively increasing the length of the column. Using columns of smaller particle size can increase the resolution of closely eluting components as well as reducing band broadening. Using a lower flow rate than the maximum recommended by the manufacturer may also lead to better resolution of closely eluting components.

Proper care of the columns and HPLC system will ensure the maximum lifetime of the system. After use, the columns and HPLC system should always be stored in a bacteriostat as recommended by the manufacturer. Worn pistons and seals should be replaced to maintain flow rate, and hence retention time reproducibility. Factors that can reduce the lifetime of a column include deposition of aggregated ma-

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terial on the column frit, introduction of air into the column, running the columns at flow rates and pressures exceeding the manufacturer's recommendations, and exposing silica-based columns to pHs >7.5.

Troubleshooting

The most common problems encountered in HPLC gel filtration are decreased sample recovery, decreased resolution of sample components, increased back pressure, and changes in retention time. The first step in troubleshooting is tracing the problem to its source: the column, sample, buffer, or instrumentation. Use a process of elimination to find the source by changing or removing one component of the system at a time.

Decreased sample recovery may be due to adsorption of the sample to the column matrix. It has been the authors' observation that some proteins tend to adhere to silica-based matrices. In particular, hydrophobic or membrane proteins may not elute from the column except in the presence of a detergent.

A decrease in resolution (due to peak tailing or broadening) or a change in retention time is often due to the formation of a void at the top of the column bed or a dead volume elsewhere in the system—e.g., a bad tubing connection between the column(s) and the detector, which creates a small mixing chamber. A void at the top of the column can be due to bed compression, introduction of air onto the column, or dissolution of silica at pHs >7.5. Bed compression can occur if the flow rate or back pressure is too high. To prevent particulate matter from clogging the frit or column matrix, buffers should be filtered and samples should be filtered or centrifuged prior to injection. Another method is to install a guard column in front of the fractionating column, which will trap any particles or precipitating proteins that are accidentally introduced into the system. Air bubbles can occur if the buffer has not been degassed properly, or they may be introduced into the system during sample injection if the sample loop is not completely filled with buffer and sample. A void may be filled using some of the same column matrix; however, care should be taken not to disturb the existing column bed.

An increase in back pressure may be due to a clogged frit. The best remedies for this are to reverse flush the column, clean the column according to the manufacturer's recommendations, or replace the end fitting. When cleaning the column, make sure the cleaning buffers are compatible with all other components of the system. The column should be run at a lower flow rate and the detector should be taken out of line. The column frit should also be changed if a visible space develops between the adaptor and the filter.

Resolution of the columns should be checked with a standard protein mix rather than with an experimental sample. An experimental sample may contain either a leading or trailing edge due to other components or impurities, such as aggregated or proteolyzed material. Routine column tests using a well-characterized standard mixture also facilitate comparative monitoring of a column's performance over the lifetime of the column.

Problems with mass spectrometry usually involve failure of the protein sample to ionize (no signal), or signal suppression due to internal standards or buffer components such as high salt or detergent. Ionization of a sample may be improved by experimenting with different matrices, sample concentrations, sample/matrix ratios, and laser power. Signal suppression due to buffer components can be remedied by dialyzing the sample into an MS-compatible buffer such as ammonium bicarbonate.

Anticipated Results

If the recombinant protein is pure and exists in a single oligomeric state with no aggregation or degradation, it is anticipated that the HPLC chromatograph will show a single, symmetrical peak. Additional peaks that elute before the protein of interest may be oligomers or aggregates of the protein, or contaminants. Peaks eluting after the protein of interest may be proteolysis products of the protein or contaminants. To identify the components, collect fractions throughout the separation procedure and analyze by another technique such as SDS-PAGE (*UNIT 10.1*), MALDI-MS (see Support Protocol 2), immunoblotting (*UNIT 10.10*), or N-terminal sequencing (*UNIT 11.10*). MALDI-MS analysis of a pure sample with the correct sequence will show a single peak with a mass that should be within 0.1% of that expected from the amino acid sequence. Final confirmation of identity can be obtained by N-terminal sequence analysis of the intact protein and/or tryptic peptide mapping (*UNIT 7.3*). Additional peaks in the spectrum may sometimes be identified by their mass (Table 7.10.3).

Time Considerations

Equilibration of the gel filtration column typically takes several hours. In some cases, equilibration may have to be performed over-

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night. An analytical HPLC gel filtration run can typically be performed in ~30 min depending upon flow rate and column length.

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Neue, 1997. See above.

A theoretical and practical guide to HPLC, including properties and selection of columns, methods development, and troubleshooting.

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CONVENTIONAL CHROMATOGRAPHIC SEPARATIONS

8

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Capillary Electrophoresis of Peptides and Proteins Using Isoelectric Buffers

Capillary electrophoresis (CE) is a high-resolution separation technique that has become a valuable tool in the analysis of many molecules. *UNIT 10.9* describes the basic concepts, instrumentation, and theory of this technology. Recent developments in the field have resulted in improvements to the procedure, and one such methodology, capillary zone electrophoresis (CZE) in amphoteric, acid buffers, is presented here.

CZE has some marked advantages over standard CE technology. For example, by using isoelectric, amphoteric buffers that have very low conductivity, CZE can accommodate high-voltage gradients (up to 800 V/cm), allowing fast separation. In addition, the acidic buffers—typically with isoelectric point (pI) values in the pH range 2.5 to 3.5—allow the use of naked, fused-silica capillaries (i.e., capillaries in which the inner surface has not been derivatized in order to suppress silanol ionization). This is an important advantage, as it drastically lowers the costs of the separation column (the cost of a fused-silica capillary soars from a few dollars/meter for an untreated material to a few hundred dollars/meter for passivated surfaces). Finally, the acidic pH values of the background electrolyte permits maximization of the net positive charge of peptide/protein analytes via protonation of Glu and Asp residues. This further accelerates the migration process and thus further reduces analysis time.

This unit presents methods for fast protein and peptide analysis in uncoated capillaries by CZE. Because the amphoteric buffers used are key to the success of the experiment, the authors have carefully studied the properties and uses of the buffers for the achievement of optimum results, and these parameters are outlined below (see Strategic Planning). Due to the acidic environment, it cannot be expected that proteins will maintain their native structure and integrity. Thus, these separations are routinely performed in 6 to 7 M urea, which accelerates the process of denaturation and helps keep the proteins in solution. As a consequence, if a protein is oligomeric, the present CZE technique will detect only the component subunits. Basic Protocol 1 details the procedure for analytical peptide separations, while Basic Protocol 2 outlines the separation of protein mixtures. An Alternate Protocol describes the detection of point mutations, a variation on Basic Protocol 2 that has applications in clinical studies. For additional information on the fundamentals of capillary electrophoresis, see *UNIT 10.9*.

STRATEGIC PLANNING

Properties of Amphoteric, Isoelectric Buffers

To perform CZE peptide/protein separation in uncoated capillaries, the buffer must have a pH close to the neutralization point of the silica wall, determined to be pH 2.3. Given an average pK value of silanols of 6.3, essentially all silanols should be protonated at pH 2.3, and thus should be unable to adsorb proteinaceous samples by an ion-exchange mechanism. Four such acidic, amphoteric buffers have been tested for use in this system: cysteic acid (Cys-A; pI 1.85), imino diacetic acid (IDA; pI 2.23), aspartic acid (Asp; pI 2.78), and glutamic acid (Glu; pI 3.22). Their properties are listed in Figure 10.13.1. The following parameters should also be considered when choosing a buffer.

Isoelectric point

The pI is not an absolute value, but a limiting pH value. At vanishing concentrations of the amphotere in solution, the pH will tend to the pI of pure water (pH 7.0); at high enough

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Contributed by Pier Giorgio Righetti, Alessandra Bossi, and Cecilia Gelfi
Current Protocols in Protein Science (1999) 10.13.1-10.13.16
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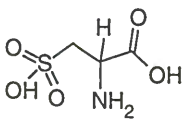
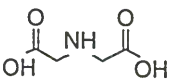
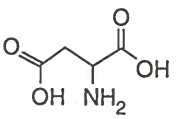
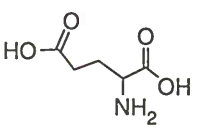
Electrolyte	Chemical formulas	pK_1	pK_2	pK_3	pI	$\lvert pI - pK_{prox} \rvert$
Cysteic acid		1.55	2.15	8.7	1.85	0.3
Imino diacetic acid		1.73	2.73	ND	2.23	0.5
Aspartic acid		1.88	3.66	9.9	2.77	0.89
Glutamic acid		2.19	4.25	9.5	3.22	1.03

Figure 10.13.1 Properties of amphoteric, acidic, isoelectric buffers. ND, not determined.

concentrations, the pH will tend to the true pI of the amphotere. For Cys-A and IDA, the given pI value corresponds to a 100 mM solution; for Asp and Glu, it corresponds to a 50 mM solution.

The ($pI - pK_{prox}$) difference

pK_{prox} indicates a protolytic group active in the proximity of the pI value. In the case of Figure 10.13.1, it refers to either pK_1 or pK_2. The absolute value of the difference between pI and pK_{prox} represents a hallmark of the amphotere. Very small values (≤1.0) indicate a very good species, possessing a high buffering capacity at pH = pI. Larger values (≥2) indicate a poor compound, unable to properly buffer at its pI value. Note that it is quite irrelevant which pK value will be displayed by the amino group in the molecule; any pK value above 6.0 will be compatible with the given buffer pIs.

Solubility

Cys-A and IDA display very high solubilities in water, whereas Asp and Glu are soluble only up to 50 mM (at 25°C).

Compatibility with organic solvents

Cys-A and IDA are compatible with a number of organic solvents, such as trifluoroethanol (TFE) and hexafluoropropanol (HFP), which impart unique electrophoretic properties to peptides. In contrast, Asp and Glu are compatible with only 10% TFE.

Conductivity

The lower the pI, the higher the conductivity of the amphotere, due to dissociation of bulk water. Thus, Cys-A and IDA should preferably be used in the presence of organic solvents (such as TFE and HFP) or 7 M urea, which, in addition to modulating the mobility of peptides and proteins, also act as conductivity quenchers.

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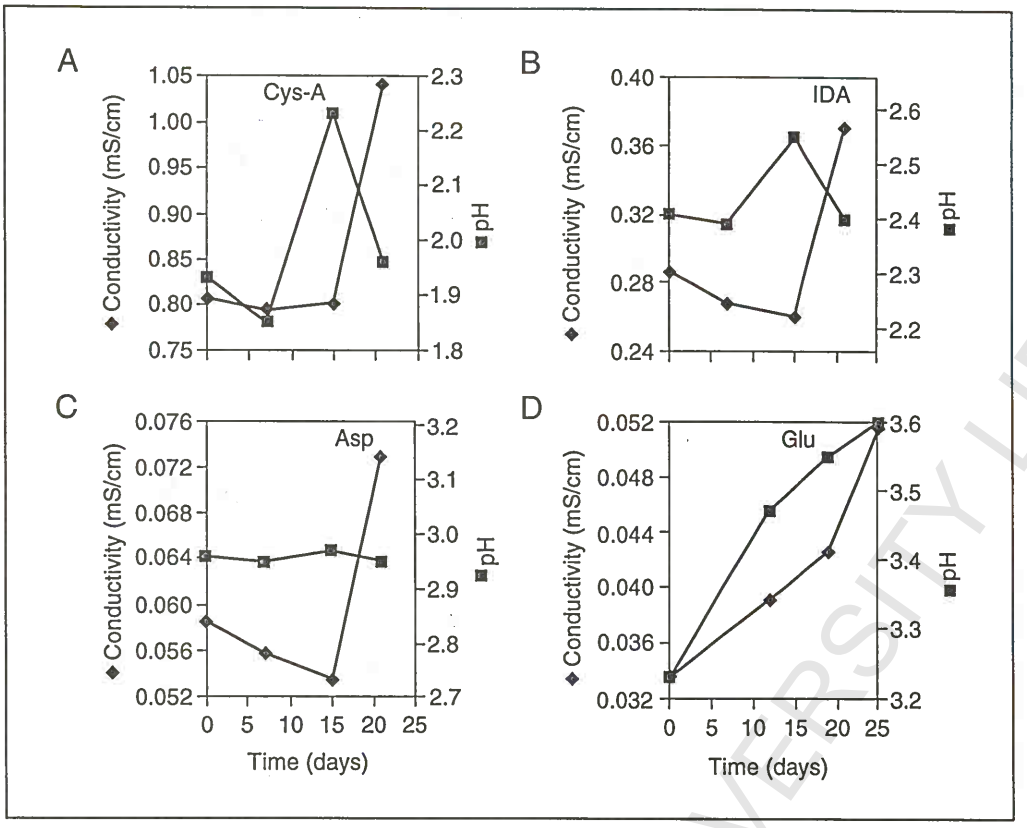

Figure 10.13.2 Conductivity and pH profiles of Cys-A (**A**), IDA (**B**), Asp (**C**), and Glu (**D**) upon aging for three weeks at room temperature. The four amphoteres were dissolved at 50 mM in plain water (unpublished data).

Addition of liquid polymers

Although the acidic pH values adopted should ensure the absence of interaction between peptides or proteins and the silica wall, in reality this risk always exists. Thus, in all buffer formulations reported, small amounts of liquid polymers are added—typically 0.5% (w/v) hydroxyethyl cellulose (HEC; mol. wt. 27,000 Da). HEC (or other liquid polymer) is dissolved at concentrations well below the entanglement threshold and does not exert any sieving of the macromolecular analytes. However, it is very effective in forming a "dynamic coating" on the silica wall, thus preventing binding of polypeptides.

Use of neutral and alkaline amphoteres

Although histidine and lysine could be excellent amphoteric ions for such separations, the authors have chosen not to address them. Histidine has been excellent for oligonucleotide separations (Gelfi et al., 1996), and the use of lysine has been reported for analysis of native proteins (Hjertèn et al., 1995). However, coated capillaries should be used with these amphoteres, considerably adding to the burden and expenses of the technique.

Long-Term Stability of Amphoteric, Isoelectric Buffers

Shelf life

An assessment of the shelf life of isoelectric buffers, dissolved as pure species in plain water as a solvent and stored at room temperature, determined that the final pH of such solutions approaches the pI value of each compound (P.G. Righetti, unpub. observ.). Because quite a number of solutions have strongly acidic pI values, they could potentially degrade at such local pH values. As indicators of potential degradation, pH and conductivity changes as a function of storage time were evaluated. Figure 10.13.2 gives these variations for four compounds, in order of increasing pI values: Cys-A, IDA, Asp, and

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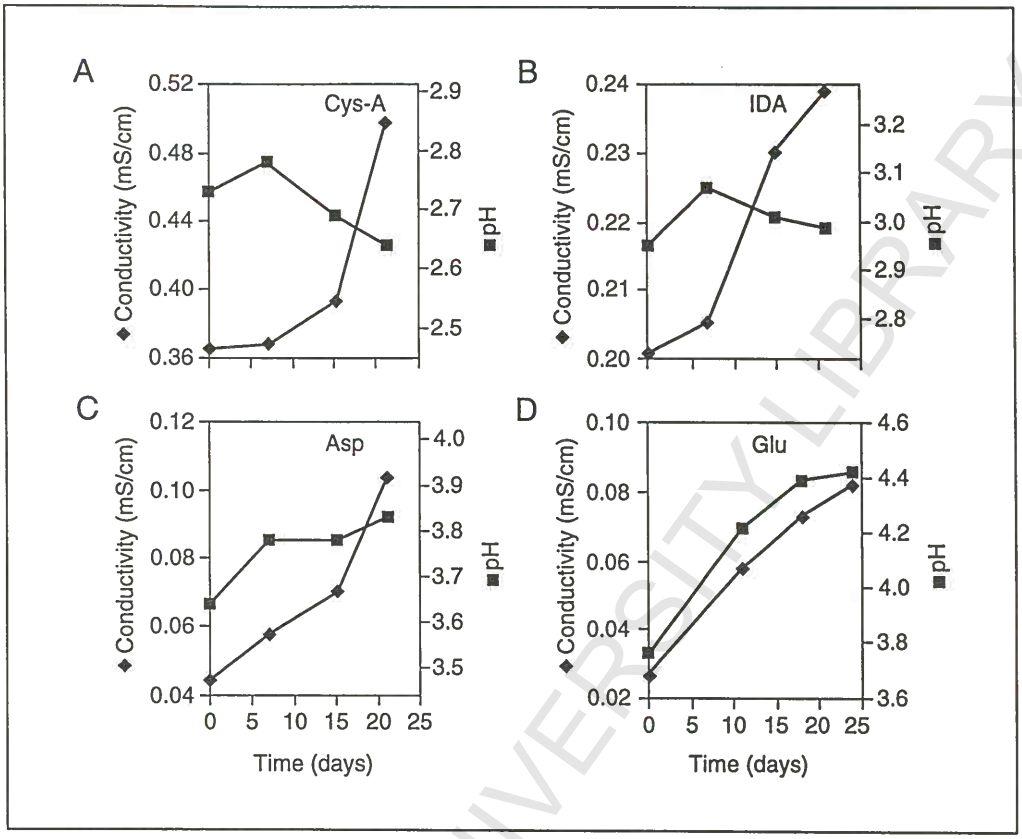

Figure 10.13.3 Conductivity and pH profiles of Cys-A (**A**), IDA (**B**), Asp (**C**), and Glu (**D**) upon aging for three weeks at room temperature. The four amphoteres were dissolved at 50 mM in an aqueous 6 M urea solution (unpublished data).

Glu. Changes were monitored for a period of up to 3 weeks. There seems to be a general trend emerging in all four compounds: the pH and conductivity of all solutions remain stable for a period of ~10 days, after which there appear to be fluctuations in both parameters. In some cases, the pH changes appear to be very minute (e.g., Fig. 10.13.2C, Asp) or to be nonmonotonic (e.g., Fig. 10.13.2A and B), so it would seem that they could be neglected. On the contrary, the conductivity increases steadily for all solutions analyzed, and this increase (in some cases quite marked) appears to be substantial after a period of 2 weeks.

Figure 10.13.3 displays the monitoring of the same parameters for the set of four compounds dissolved in 6 M urea. Here too it is difficult to draw any conclusions from the shape of the pH curves: they either fluctuate or give minimal absolute changes with time, except for Asp and Glu, which show a steady increase in pH for a total of 0.2 and 0.6 pH units, respectively, over a period of 3 weeks. Conductivity changes, on the other hand, are quite marked and appear to increase constantly for all buffers studied. In addition, the onset of such conductivity increments occurs quite early compared to the same solutions in the absence of urea (typically after only a few days).

Making and using new stocks of buffers

Another practical point of interest to users of CZE is how often one should replenish the anolyte and catholyte solutions, so as to avoid electrode polarization as well as marked changes of buffer pH and conductivity due to buffering ion and counter ion depletion. This point, in principle, should not be critical when using isoelectric buffers; their depletion at either electrode should be negligible, because they should not bear a net charge. Figure 10.13.4 explores potential pH and conductivity variations as a function of time of operation of the CZE instrument under a constant electric field of 20,000 V, using

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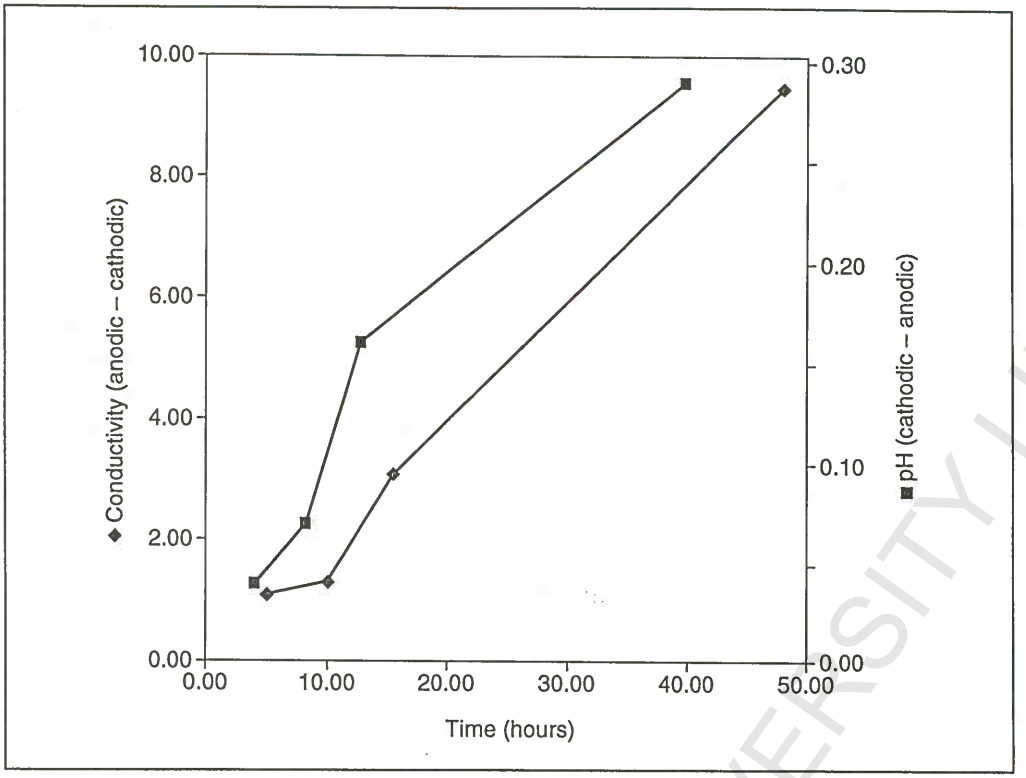

Figure 10.13.4 Conductivity and pH profiles of 40 mM Asp with 6M urea and 0.5% HEC, used for up to 50 hr as anolyte and catholyte. Electrophoretic conditions: 20 kV at room temperature without any change of electrolytes. The data are expressed as the difference in conductivity and pH measured in the two electrodic vessels (unpublished data).

Asp as the sole buffering species in all compartments (anode, cathode, capillary lumen). The data are expressed as the difference in pH and conductivity between the two electrode vessels. Note that in continuous operation the pH increases at the cathode, whereas the conductivity increases at the anode. This means that there must be a net transport of Asp from the cathodic to the anodic vessel. If that is the case, the pH should keep increasing at the cathode and decreasing at the anode. Decrements of pH in the anodic vessel should bring about increments of conductivity. This is to be expected. In fact, at progressively more acidic pH values, a fraction of the amphotere should be nonisoelectric in order to neutralize the excess positive charges brought about by bulk water ionization. For example, in a solution of Asp at pH 3.0, there must be 1 mM proton concentration, which must be neutralized by a 1 mM fraction of Asp being nonisoelectric and negatively charged. This excess, nonisoelectric, molar fraction will constantly migrate towards the anode, creating an imbalance in the concentration of Asp between the two electrodic vessels. This phenomenon can be neglected during the first 10 hr of operation, but after that it becomes quite appreciable.

Practical approaches

Considering the information given above, the following guidelines should apply to CZE in isoelectric, acidic buffers:

1. Buffers, especially if made in the presence of urea, should be prepared fresh every week, stored at 4°C, and then discarded.
2. Anolyte and catholyte solutions should be refreshed after each run when using the most acidic buffer (Cys-A). As the pI increases, the electrodic solutions can be progressively used for a few sequential runs before replenishing (e.g., in case of Asp, buffer can be used for a full day of operation before being discarded).

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3. As a corollary to the above, monitoring the electrodic vessels for pH and conductivity changes will give a sure indication of when to refresh these solutions.

BASIC PROTOCOL 1

ANALYTICAL PEPTIDE SEPARATIONS

CZE is particularly useful for generating peptide maps (fingerprinting) from complex mixtures, such as the products of proteolytic digestion (Bossi and Righetti, 1997; Capelli et al., 1997; Righetti and Nembri, 1997; McLaughlin et al., 1998). In this procedure, the peptide mixture to be analyzed is placed in a sample vial at the cathodic end of the capillary column. The anodic reservoir contains running buffer. The sample is loaded into the capillary either electrokinetically or by pressure or vacuum, and then the sample vial is replaced by running buffer and electrophoresis begins. The following protocol describes the use of a Bio-Rad Bio Focus 3000 instrument using low-pressure sample injection. Other instruments are capable of similar separations when operated according to the manufacturer's instructions.

Materials

Peptide mixture (e.g., β-casein digestion; see recipe)
0.1 M NaOH
Asp buffer: 50 mM aspartic acid (pH = pI = 2.77) containing 0.5% (w/v) hydroxyethyl cellulose (HEC; mol. wt. 27,000 Da, conductivity at 25°C 0.42 mS/cm)
Capillary: 37-cm length (32.4 cm to the detection window); 75-µm i.d., 375-µm o.d. (Polymicro Technologies)
Capillary zone electrophoresis (CZE) instrument (Bio Focus 3000, Bio-Rad, or equivalent)

1. Precondition a new capillary by flushing it with the following solutions, and then store the column in Asp buffer at 25°C.

   1 M NaOH (1 hr)
   H_2O (15 min)
   1 M HCl (2 hr)
   H_2O (15 min)
   0.1 M NaOH at high pressure (30 min)
   H_2O (5 min)
   Asp buffer (30 min).

   When changing to a different separation buffer, equilibrate the capillary for 4 hr with the new buffer by flushing at high pressure for 10 min and monitoring the baseline for 1 hr.

   The preconditioning step is essential if a new capillary is being used. If the capillary has already been properly equilibrated in separation buffer, it is best to avoid any conditioning with solutions of widely different pH, so as not to disturb the silica surface and alter its ionization properties.

   The capillary should be stored no more than 5 days

2. Load 10 to 20 nl peptide mixture using pressure injection for 0.2 to 1 sec at 5 psi according to manufacturer's instructions.

3. Separate the peptide mixture using CZE instrument according to manufacturer's instructions and using the following conditions:

   Electrolyte: Asp buffer used as anolyte, catholyte, and for filling the capillary
   Detector wavelength: 214 nm
   Temperature: 25°C
   Voltage: 400 to 600 V/cm
   Run time: up to 20 min.

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4. Between runs, rinse the column with the same Asp buffer used for separations.

When using the same capillary for a series of separations of the same type of sample, store the column in running buffer at room temperature. The capillary can be stored in this state for no more than 5 days.

Figure 10.13.5 gives an example of the development of these peptide maps at 400 or 600 V/cm. Note that in this last case the entire map is developed in only 11 min. The same separation, when using a standard high conductivity buffer (80 mM phosphate, pH 2.0) takes ~70 min, with severe loss of resolution due to diffusional peak spreading.

SEPARATION OF PROTEINS IN ACIDIC ISOELECTRIC BUFFERS

BASIC PROTOCOL 2

Knowledge of the pIs of the components of the mixture would greatly help in selecting an appropriate run buffer. One capable of maximizing differences in the charge-to-mass ratio and ensuring that the proteins will all migrate towards the detector window. If the pI of a protein is unknown, separations can be achieved by using either a very alkaline or a very acidic buffer. However a coated column must be utilized with very alkaline buffers, so as to suppress any interaction of the analyte with the capillary wall. Therefore, very acidic buffers are preferred, as they should effectively quench adsorption of proteins onto the silanols. There is a catch to this strategy, however—in such an acidic environment proteins will most likely unfold and denature, due to disruption of the hydrogen bonds. Such denaturation could also induce protein aggregation and precipitation. The addition of 6 to 7 M urea in the background electrolyte ensures full denaturation while maintaining solubility of the analyte. Since the addition of 7 M urea will increase the apparent pH of the background electrolyte by ~1 pH unit, it is suggested that low pI buffers, such as IDA (pI 2.33) or Asp (pI 2.77), be used rather than Glu (pI 3.22). Please note that for oligomeric proteins, it is the subunits that will be separated (unless they are held together by disulfide bridges). The following protocol is based on a procedure routinely used for separation of wheat gliadins (Capelli et al., 1998).

Materials

Protein sample (e.g., gliadin extraction; see recipe)
0.1 M NaOH
70% (v/v) ethanol
Asp/urea buffers: 40 mM aspartic acid (pH = pI = 2.77), containing 0.5% (w/v) hydroxyethyl cellulose (HEC; mol. wt. 27,000 Da) and 7 M *and* 5 M urea (apparent pH 3.9; conductivity at 25°C: 0.7 mS/cm; both values refer to 7 M urea solutions)
Capillary: 30-cm length (25.4 cm to the detection window), 50-µm i.d., 375-µm o.d. (Polymicro Technologies)
Capillary zone electrophoresis (CZE) instrument (Bio Focus 3000, Bio-Rad, or equivalent)

1. Precondition a new capillary by flushing it with the following solutions. After equilibration, store in water at 25°C.

1 M NaOH (1 hr)
H_2O (15 min)
1 M HCl (2 hr)
H_2O (15 min)
0.1 M NaOH at high pressure (30 min)
H_2O (5 min)
Asp buffer/7 M urea buffer (30 min)

When changing to a different separation buffer, equilibrate the capillary for 4 hr with the new buffer by flushing at high pressure for 10 min and monitoring the baseline for 1 hr.

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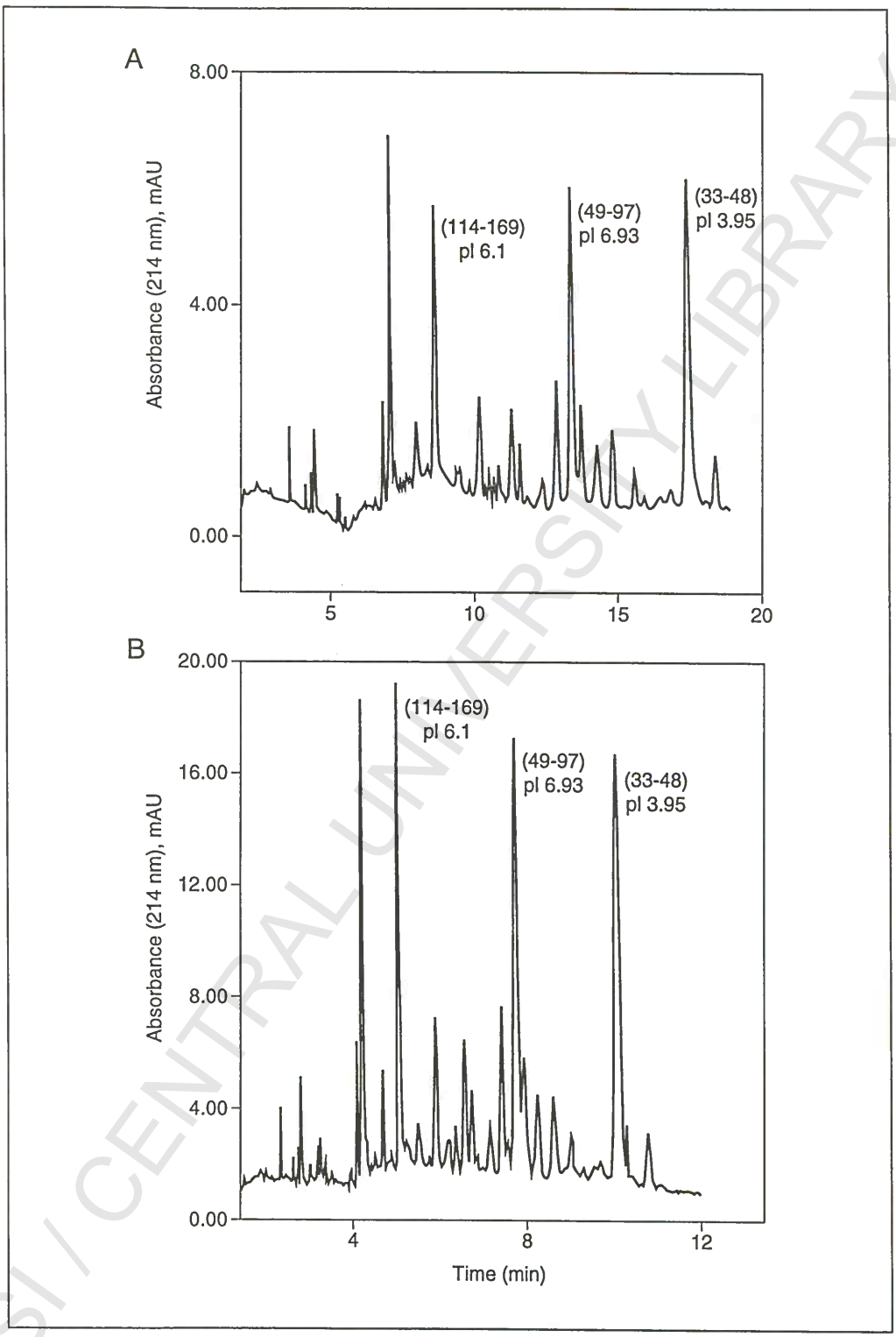

Figure 10.13.5 CZE of tryptic digests of β-casein in a 37-cm × 100-μm-i.d. capillary bathed in 50 mM isoelectric Asp (pH approximating the pI value of 2.77) with 0.5% HEC. (**A**) 400 V/cm (current: 33 μA); (**B**) 600 V/cm (current: 58 μA). The three major labeled peaks are (from left to right) the pI 6.1 fragment (β-CN 114-169); the pI 6.9 fragment (β-CN 49-97) and the pI 3.95 fragment (β-CN 33-48). CN, casein. Reproduced from Righetti and Nembri (1997), with permission from Elsevier.

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The preconditioning step is essential if a new capillary is being used. If the capillary has already been properly equilibrated in separation buffer, it is best to avoid any conditioning with solutions of widely different pH, so as not to disturb the silica surface and alter its ionization properties.

2. Dilute the protein sample 1:5 (v/v) with 70% ethanol to give a final concentration of 2 mg/ml.
3. Fill the uncoated capillary with Asp/urea buffer containing 7 M urea. Fill the anode and cathode reservoirs with Asp/urea buffer containing 5 M urea.
4. Inject the sample hydrodynamically (e.g., 3 sec at 1 psi) according to manufacturer's instructions.

   For a 2 mg/ml sample, 0.2 sec at 5 psi is sufficient.

5. Separate using the following conditions:

   Electrolyte: Asp/urea buffer containing 5 M urea used as anolyte and catholyte, and containing 7 M urea for filling the capillary
   Detector wavelength: 214 nm
   Temperature: 25°C
   Run voltage: 800 to 1000 V/cm
   Run time: 15 min.

   Figure 10.13.6 gives an example of a CZE run of four different cultivars of durum wheat, and Figure 10.13.7 gives an analogous separation for four cultivars of bread wheat. Note that these separations are typically over in 10 to 12 min. In contrast, the same separations performed in standard 100 mM phosphate buffer, pH 2.5, last for >30 min.

6. Between runs, rinse column with the same Asp/urea buffer used for separations.

SEPARATION OF POINT MUTATIONS IN POLYPEPTIDE CHAINS

ALTERNATE PROTOCOL

Detection of point mutations is important in identifying several congenital defects of major clinical interest. For example, in the case of hemoglobinopathies, >600 defective variants of hemoglobin (Hb) have been reported. Among those, approximately one third involve a neutral-to-charged amino acid substitution, easily detectable by routine electrophoretic techniques, since they bring about a variation of the net charge of the expressed polypeptide. However, approximately two thirds represent the so-called electrophoretically silent mutations, in that they involve a neutral-to-neutral amino acid substitution. In this last case, not only is there no appreciable change in net charge, but the variation of polypeptide mass is also so minute as to escape detection by SDS-PAGE (*UNIT 10.1*). It has recently been demonstrated that addition of a neutral surfactant can in fact induce separation of the wild-type and mutant chains, probably by hydrophobic interaction of the more hydrophobic mutant with the detergent micelle, via a mechanism similar to micellar electrokinetic chromatography (Terabe et al., 1985). This is a protocol the authors routinely utilize for separation of point mutations in human globin chains (Righetti et al., 1998a; see also Regnier and Lin, 1998; Righetti et al., 1998b).

Additional Materials (also see Basic Protocol 2)

Protein sample (e.g., globin chain preparation; see recipe)
IDA buffer: 50 mM imino diacetic acid (pH = pI = 2.23), containing 0.5% (w/v) hydroxyethyl cellulose (HEC; mol. wt. 27,000 Da) and 7 M *and* 5 M urea (apparent pH 3.2; conductivity at 25°C: 176 µS/cm)
Uncoated capillary, 33-cm length (26 cm to the detection window), 50-µm i.d., 375-µm o.d. (Polymicro Technologies)
Capillary zone electrophoresis (CZE) instrument (Bio Focus 3000, Bio-Rad, or equivalent)

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1. Condition the capillary (see Basic Protocol 2, step 1).
2. Fill the uncoated capillary with IDA buffer solution containing 7 M urea buffer. Fill the anode and cathode reservoirs with IDA buffer solution containing 5 M urea.

   If studying neutral point mutations, add 2% reduced Triton X-100 to the buffer.
3. Inject the sample hydrodynamically (e.g., 3 sec at 1 psi or 0.2 sec at 5 psi) according to manufacturer's instructions.
4. Separate using the following conditions:

   Electrolyte: IDA buffer containing 5 M urea used as anolyte and catholyte, and containing 7 M urea for filling the capillary
   Detector wavelength: 214 nm
   Temperature: 15°C
   Run voltage: 600 V/cm
   Run time: 10 min.

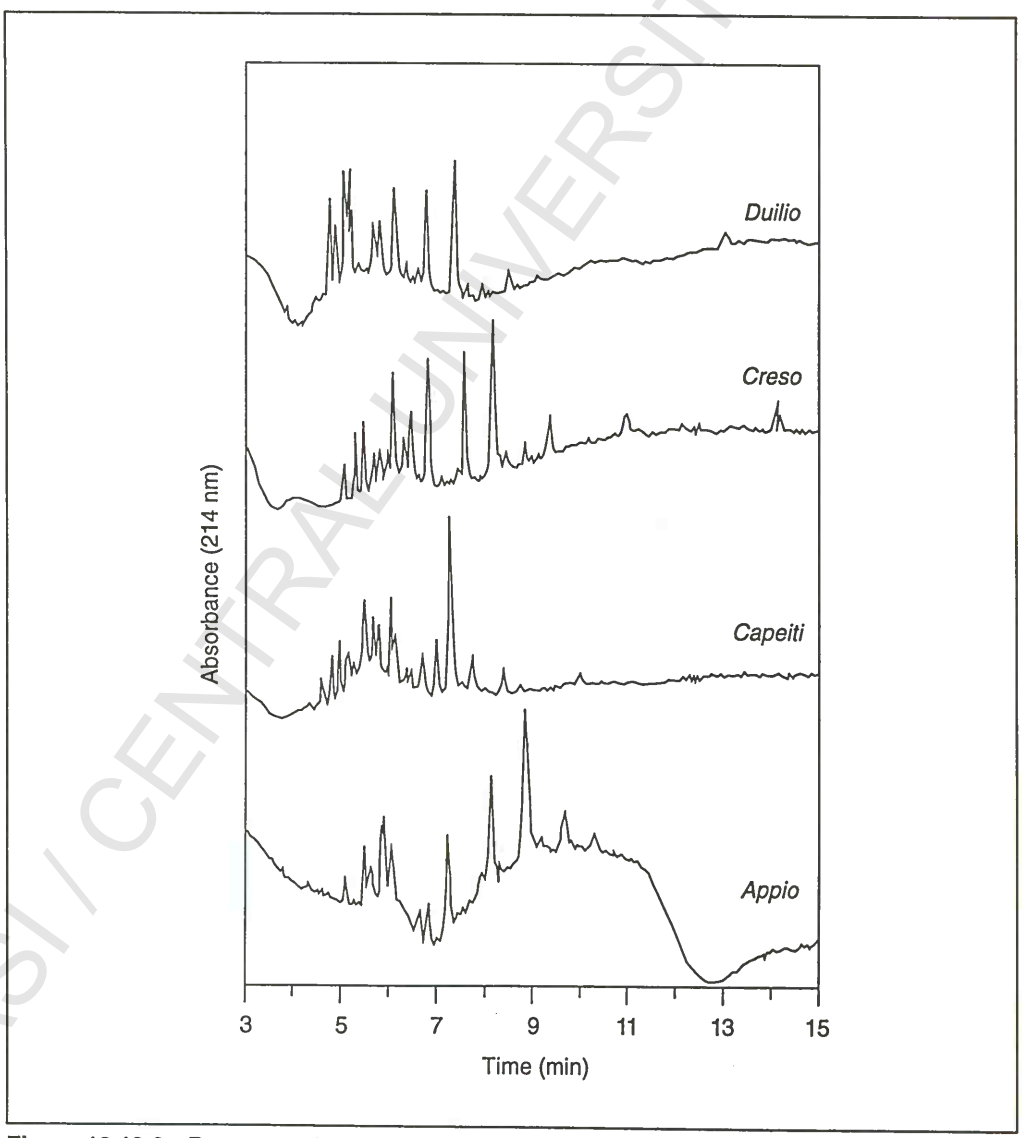

Figure 10.13.6 Representative CZE run of gliadins, obtained by sequential extraction of flour from four different cultivars of durum wheat. Run performed in 40 mM Asp with 7 M urea and 0.5% HEC (apparent pH 3.9). Conditions: 50-µm-i.d. × 30-cm-long capillary, run at 1000 V/cm, room temperature. From Capelli et al. (1998), with permission from Wiley-VCH.

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Figure 10.13.8 gives an example of a separation of a β-chain mutant in both control (in the absence of surfactant) and background electrolytes containing 2% (v/v) reduced Triton X-100. Notice, in the lower panel, the splitting of the two β-chains, which coelute in the absence of surfactant. Note that these separations are typically over in 6 to 7 min. The same separations performed in standard 100 mM phosphate buffer, pH 2.2, take ~20 min.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

β-casein digestion

Dissolve 10 mg/ml β-casein in 50 mM 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS) buffer, pH 8.2. Add trypsin at a trypsin/β-casein ratio of 2% (w/w). Allow to hydrolyze for 4 hr at 50°C. Stop the reaction by adding 4 M acetic acid to pH 4.0. Store the tryptic peptide digest up to 6 months at –20°C.

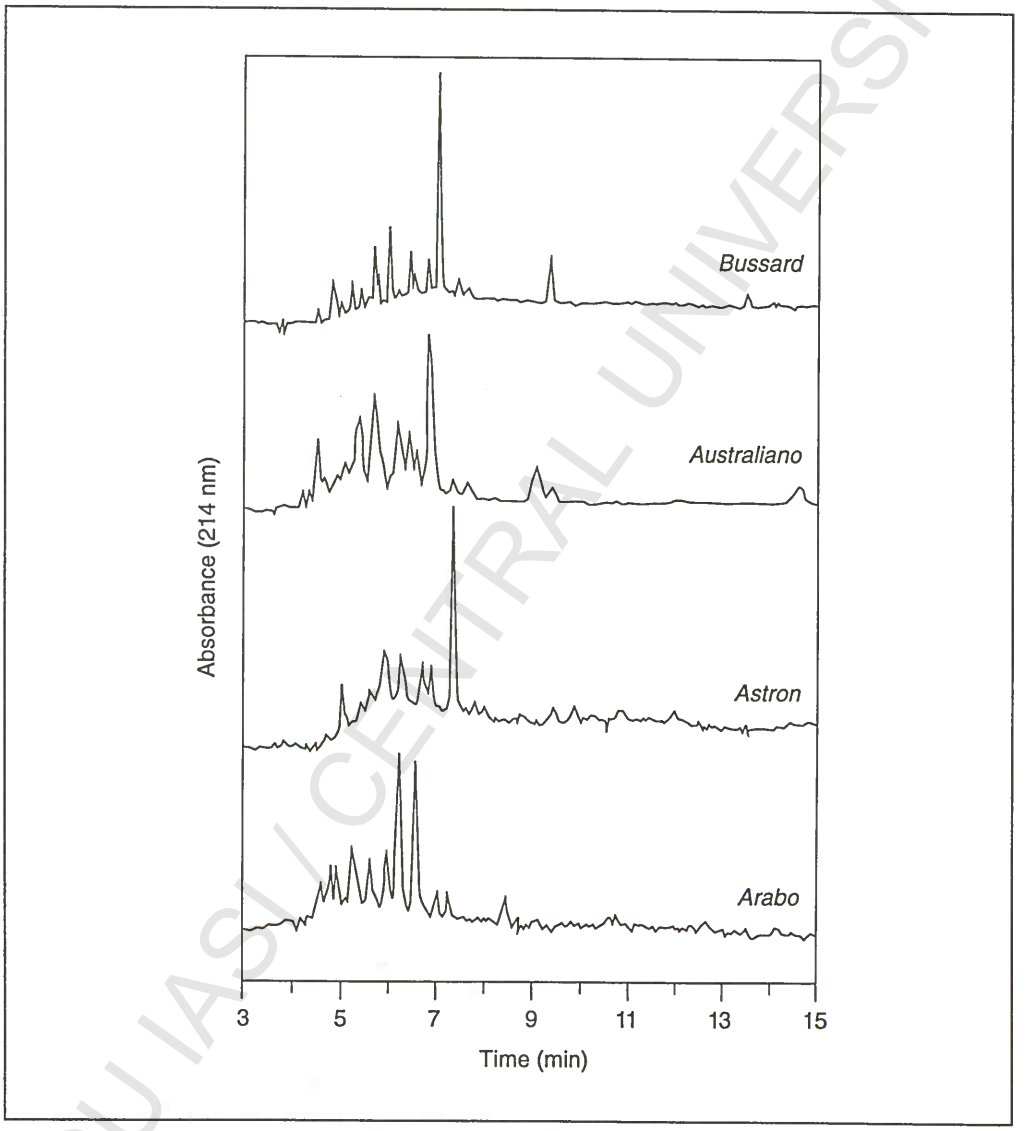

Figure 10.13.7 Representative CZE run of gliadins, obtained by sequential extraction of flour from four different cultivars of bread wheat. Run performed in 40 mM Asp with 7 M urea and 0.5% HEC (apparent pH 3.9). Conditions: 50-μm-i.d. × 30-cm-long capillary, run at 1000 V/cm, room temperature. From Capelli et al. (1998), with permission from Wiley-VCH.

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Gliadin extraction

In the direct extraction, extract 30 mg bread or durum wheat flour while vortexing for 30 min at room temperature with 0.5 ml cold 70% (v/v) aqueous ethanol. Centrifuge for 15 min at 11,000 × *g*, 20°C. Use the supernatant, containing mostly gliadins (10 mg/ml), as is for CZE analysis. Store up to 6 months at –20°C.

Globin chain preparation

Wash red blood cells in physiological saline (0.9% NaCl), lyse in distilled water, and pellet the resulting "ghosts" by centrifuging 5 min at 5000 × *g*, 4°C. Add 20 µl concentrated hemoglobin (Hb) solution dropwise to 1.5 ml acetone containing 2% (v/v) HCl (at room temperature) in a microcentrifuge tube. Centrifuge again as before, discard the supernatant and resuspend the pellet in water. Repeat the centrifugation again, discarding the supernatant, in order to obtain a white globin powder. Dry the

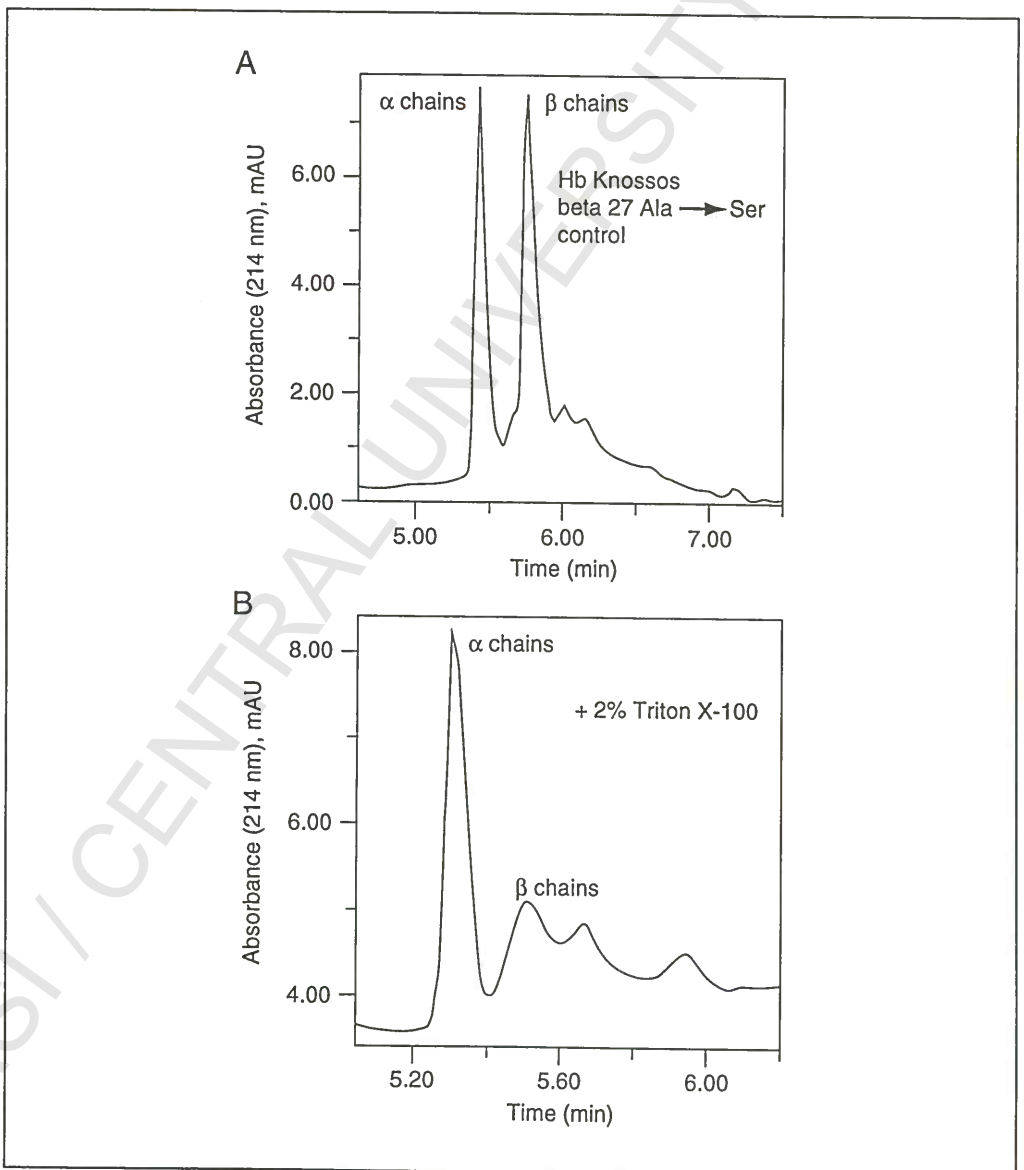

Figure 10.13.8 CZE separation of hemoglobin Knossos (β27 Ala → Ser). (**A**) Control run; (**B**) buffer added with 2% reduced Triton X-100. CZE conditions: 50 mM IDA with 7 M urea and 0.5% HEC (apparent pH 3.2). Capillary: uncoated, 33-cm (length to detector 26 cm) × 50-µm-i.d. × 375-µm-o.d. Run at 600 V/cm (25 µA current) at 15°C. from Righetti et al. (1998a), with permission from Wiley-VCH.

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pellet in a Speedvac evaporator. Prior to analysis, dissolve the globin powder at a concentration of 2 mg/ml in background electrolyte containing 2% (v/v) 2-mercaptoethanol and only 5 (instead of 50) mM IDA. Store up to 6 months at –20°C.

COMMENTARY

Background Information

CZE of proteins and peptides has usually been performed either at very acidic (pH 2 to 2.5) or rather alkaline (pH 9 to 10) pH values. Under alkaline conditions, a coated capillary has to be used, in order to quench the electroendoosmotic flow (EOF) and to minimize residual adsorption to fully ionized silanols. Under acidic conditions, a naked capillary can be used, but the very high conductivity of such acidic buffers (notably phosphate), allows only low-voltage gradients (e.g., maximum 100 V/cm) with concomitant very long analysis times.

The first report on the use of amphoteric buffers, used as the sole background electrolyte at pH = pI, came from Hjertèn's group in 1995 (Hjertèn et al., 1995). This group could separate albumin and transferrin in only 40 sec using 15-cm-long capillaries at 2000 V/cm in low-conductivity polymeric buffers (pH 8.6). In 5 mM Lys (pH = pI = 9.7), a mixture of different protein markers (soybean trypsin inhibitor, ovalbumin, conalbumin, and myoglobin) could also be separated in 240 to 40 sec at voltage gradients ranging from 330 to 2000 V/cm. However, while Hjertèn's procedure solved the problem of fast analysis times, it had to adopt capillaries with an inner coating of polymeric material (usually polyacrylamide) covalently anchored to the silanols.

The first article published dealing with the use of amphoteric, isoelectric buffers was that of Mandecki and Hayden (1988), on the use of His as the sole buffering ion in slab gel electrophoresis of DNA. Although this methodology appeared in 1988, it went largely unnoticed until it was rediscovered by Hjertèn's group. The authors have also adopted isoelectric His in CZE for the fast separation of antisense oligonucleotides in liquid-sieving polymers (Gelfi et al., 1996). The main difference between previous data and the present technique resides in the use of acidic, isoelectric buffers, with all the benefits inherent to this methodology, such as fast analysis times, high resolution, and the ability to use untreated fused-silica capillaries.

Critical Parameters

For separation of peptides (see Basic Protocol 1), it is generally not necessary to add urea, as these fragments are well soluble in plain water solvent because of their relatively small size and good hydrophilicity. One exception would be peptides obtained from hydrophobic (e.g., membrane) proteins—these samples run the severe risk of aggregating and/or precipitating. In this case, addition of urea, possibly also combined with surfactants, is recommended. If detergents are used, neutral or zwitterionic species (such as reduced Triton X-100 or CHAPS) work best. Do not use Nonidet P-40 or other detergents that contain aromatic rings, as their absorbance at 200 nm will lower the sensitivity of detection.

In mixed hydro-organic solvents of the water/urea type, it should be remembered that, due to the heat developed in a capillary unit and to the strong air streams in equipment that uses forced-ventilation cooling, there is a strong risk of solvent evaporation in the two electrode vessels. Urea will concentrate and crystallize, blocking the inlet of the capillary. Thus, as a safety precaution, only 5 M urea should be added to the electrode vessels when using buffers containing urea. For the running buffer in the capillary, the urea concentration can be increased to 7 M urea, but not higher than that. With high levels of urea, be sure that the extremities of the capillary are always kept inserted in a vessel containing liquid. When left in the air, the solvent will quickly evaporate from the capillary tips and urea will crystallize.

For protein separations (see Basic Protocol 2 and see Alternate Protocol), there is often the risk that small amounts of protein will be adsorbed onto the silica wall. After repeated injections, there might be a significant alteration of the silica surface, leading to irreproducible results and even to spurious peaks. A simple solution is to dispose of the capillary and start with a new one. However, if the same capillary has to be reused, there are several procedures for regenerating the silica wall. In one (drastic) treatment, the capillary can be rinsed with 0.1 N (or even 1 N) NaOH. Alkaline treatments are very effective in removing and solubilizing proteinaceous material and most adsorbed impurities. Unfortunately, such a drastic procedure will ionize most of the surface silanols and deeply alter the surface properties of the silica

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wall. In addition, because alkaline treatments are abrasive, part of the silica surface can be dissolved. As a result, it might be very difficult to recondition the capillary wall to the original properties and obtain reproducible results in electroendoosmotic flow and transit times.

A better way to remove adsorbed material is by rinsing the capillary with running buffer containing micellar SDS (e.g., 30 mM SDS). However, if the running buffer has too low a pI, the SDS micelles might not be effective—in this case, dissolve the SDS in Good's buffers (e.g., MES or MOPS, titrated at pH 6.8). The running buffer/SDS solution is placed at the cathode and the SDS micelle is transported electrophoretically into the capillary. The migrating SDS micelles act by sweeping any silica-bound material, which will be adsorbed into the micelle and migrate with the SDS front. If this operation is conducted under UV monitoring, a peak of desorbed material will be seen at the passage of the SDS micelles. This electrophoretic desorption can be continued for 30 min under constant electric field. This process is the electrophoretic analog of frontal chromatography. After this step, the SDS micelles can be removed by rinsing with water and then the capillary can be reconditioned by purging with the chosen isoelectric running buffers (10 column volumes; see Basic Protocol 1, step 1), in the presence of the proper additives, if any.

It appears that the two best buffers, in general, are IDA and Asp. Cys-A is strongly acidic and does not offer particular advantages over such a strongly conducting buffer as phosphate, titrated at pH 2.0 to 2.5. Cys-A is best used in presence of conductivity quenchers, such as urea and/or organic solvents (TFE and HFP) that are compatible with this buffer and with bulk water at any concentration (although, typically, no advantage is obtained at concentrations greater than 40%, v/v). Cys-A seems to be particularly attractive when analyzing small (di-, tri-, and tetra-) peptides that bear the terminal carboxy and amino groups as their only charged groups. In fact, such peptides are isoelectric over a broad pH range (typically at least 4 to 8), so that either strongly acidic or strongly basic solutions are needed in order to provide sufficient charge for a fast separation.

Glu is also an interesting buffer, because its higher pI (3.22, especially when in presence of urea, apparent pH 4.3) allows modulation of the net charge of large peptides, due to partial deprotonation of Asp and Glu residues. Thus, in general, the titration curves of polypeptides tend to fan out at and above pH 4, allowing mobility modulation and increased selectivity. The risk with Glu, however, is that at this higher pH (especially in the presence of 7 M urea) polypeptides might bind to ionized silanols, resulting in loss of resolution. Thus, when adopting Glu as a background electrolyte, one should perform preliminary runs in order to identify potential interaction of the silica wall with the components of the protein mix under analysis.

Troubleshooting

Potential mechanical and/or electrical problems that might be encountered during instrument use are usually addressed in the manufacturer's manual. Therefore, this discussion will focus on problems encountered with the sample and background electrolyte. The most common problems encountered in CZE are discussed below.

Loss of current during a run

A typical problem is the sudden or gradual loss of electric contact during a run. Sudden loss of electric contact could be due to a breaking of the capillary or the trapping of a large air bubble spanning the entire lumen of the capillary. Gradual loss of current could be caused by small air bubbles that develop during the run (e.g., due to excess power forcing the solution to outgas), which adhere to the silica wall but are too minute to completely obstruct the lumen. During the run, more air bubbles could develop and further reduce the lumen available to the passage of electric current. On a long run, such tiny air bubbles could coalesce and suddenly break the current (remember that air bubbles can be negatively charged due to adsorption of ions in their diffuse double layer, and thus can migrate in the electric field). Bubbles can be avoided by thoroughly degassing all solutions used in the instrument (including the reservoirs). The authors often adopt a double degassing procedure: under vacuum and also by sonication. It is also a good procedure to check the total applied electric power and see that it does not exceed the power recommended by the manufacturer.

One should also make sure that the sample vial contains enough sample to cover the extremity of the capillary at the injection site, so as to prevent injection of air into the column. Remember that, although the sample volume injected in the capillary is minute (typically 5 to 20 nl), for proper immersion of the capillary end in the analyte solution the vial should contain 10 µl. In addition, the sample vials can

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dry out during a long run, especially when performing sequential runs and placing a large number of samples in the carousel. One would then notice that the later samples give exaggeratedly huge peaks due to excessive liquid evaporation. In order to avoid that, when programming a sequence of runs (e.g., for overnight analyses), the authors have adopted a neat safety procedure, consisting of overlaying the analyte solution with a layer of light paraffin oil. This oil will float on top of the liquid layer of analyte, effectively sealing it from the air and completely preventing evaporation.

Arcing

CZE instruments can arc during a run. This is often caused by salt deposits or moisture on the high-voltage electrode stations. In instruments using a free capillary, not encapsulated in a cartridge, moisture can be deposited all along the capillary's outer surface, causing creeping current on its surface. This can be particularly true in a hot and humid summer environment in the absence of proper air conditioning and dehumidification. A proper cure would be to purge the capillary chamber with dry inert gas and to place Drierite bags in the capillary holding compartment.

Column plugging

The column can plug because of salt deposition as the liquid inside dries out. This is particularly true when using urea-laden buffers. As a safety precaution, if no further use of the capillary is expected at the end of a working day, the column should be emptied and stored overnight in plain water or in urea-free buffer. In general, washing the column with water dissolves the plug if a standard buffer is used; however, in the case of urea-containing buffers, it might be necessary to discard the capillary if extensive deposition of urea has occurred in a substantial portion of its length. Proteins too can precipitate during a run and plug the column. Using lower protein concentrations usually prevents this type of plugging.

Sudden loss of sample resolution

When using very acidic isoelectric buffers (e.g., Cys-A), poor resolution can be caused by ion depletion at one electrode due to electrophoretic migration of the nonisoelectric molar fraction. When this occurs, most of the voltage gradient falls on the electrode vessel depleted of ions, with consequently poor or no migration of the analyte components. Check the electrode pH and conductivity, and refresh the buffer.

Sample tailing

Sample tailing could be due to adsorption of sample ions to ionized silanols. It might occur in two particular cases: when using proteins (peptides are generally too small to elicit substantial binding to the silica wall) and when using progressively higher pI amphoteres, such as Glu. Adsorption of polypeptides to the capillary can be demonstrated by frontal analysis, i.e., by making a blank run in which SDS micelles are added to the cathodic vessel. On their migration to the anode, the SDS micelles will sweep away bound proteins and a sharp sample peak will be detected. The remedy is to adopt lower pI amphoteric buffers and/or increase the amount of sieving liquid polymer (HEC, which should be used below 1%, as it will give too high a viscosity).

Unreproducible transit times

A possible cause of unreproducible transit times could be too short an equilibration period of the capillary in a given buffer. Let the capillary equilibrate overnight, instead of only 4 hr, with frequent periods of pumping the buffer through the column. This will also favor deposition of the HEC polymer onto the inner capillary surface, a process called dynamic coating. Another cause could be a drastic washing step with alkaline solutions (e.g., 0.1 N NaOH). This will ionize a large number of silanols and deeply alter the charge state of the surface. It might then take extensive washings in the original acidic buffer solution in order to reestablish the proper starting surface conditions.

Often, the biggest offender is accumulation of adsorbed material from the sample plug. This material might not necessarily be the protein or peptide of interest, but some impurities in the sample matrix (e.g., lipids). One remedy to it is to try to pre-purify the sample solution, e.g., by a lipid-extraction step. The other remedy is to wash the capillary with a front of SDS micelles, which will quite efficiently desorb bound material by forming mixed micelles.

Anticipated Results

Results of separations obtainable with the experimental conditions outlined here are discussed with each of the protocols (also see Figs. 10.13.5-10.13.8).

Time Considerations

As a virtue of the high voltage gradients that can be applied in isoelectric buffers, the total analysis time is much reduced compared to analogous techniques in conventional, non-

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isoelectric buffers. For example, in Asp buffer at the standard voltage of 600 V/cm in a 37-cm-long capillary, analysis is usually over in 10 to 12 min. The total analysis time, including sample loading, running, and capillary regeneration prior to the following run, is typically on the order of 15 min.

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CHAPTER 19
Identification of Protein Interactions

INTRODUCTION

Protein interactions are intrinsic and vital to many cellular processes. In recent years many new techniques have been developed for the study of this important topic; the review by Phizicky and Fields (1995) provides an excellent general overview of this subject. This chapter describes biochemical, physical, and genetic methods that are used to detect specific protein interactions. A companion chapter in preparation will present a series of complementary methods that are used to provide quantitative information about protein interactions, such as the strength and kinetics of binding.

This chapter begins with brief overview of the analysis of protein interactions in which some of the basic kinetic and thermodynamic concepts are described (*UNIT 19.1*). This is followed by a presentation of one of the most widely used approaches for detecting protein interactions, the yeast two-hybrid/interaction trap system (*UNIT 19.2*). In this genetic method transcriptional activity is used as a measure of protein interaction.

The technique of interaction cloning or expression cloning based on phage display can be used to identify and clone genes that interact with known proteins of interest. *Escherichia coli* filamentous phages can express fusion proteins bearing foreign peptides on their surface. These peptides are accessible to bind with antibodies or other proteins to which they have some affinity. *UNIT 19.3* details phage expression cloning to identify phage fusion proteins that interact with known targets or bait proteins. One important characteristic of this approach is that the phage fusion protein is directly associated with its gene due to its packaging in a single phage, making it possible to immediately sequence the gene and thereby generate one or more consensus sequences of the bound peptides.

Future plans for this chapter includes a unit on surface plasmon resonance (SPR), another useful method for discovery and detection of protein interactions that is gaining in popularity.

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Phizicky, E.M. and Fields, S. 1995. Protein-protein interactions: Methods for detection and analysis. *Microbiol. Rev.* 59: 94-123.

Paul T. Wingfield

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Theoretical Aspects of the Quantitative Characterization of Ligand Binding

Living organisms grow, differentiate, reproduce, and respond to their environment via specific and integrated interactions between biomolecules. The investigation of molecular interactions therefore constitutes a major area of biochemical study, occupying a ubiquitous and central position between molecular physiology on the one hand and structural chemistry on the other. While specificity resides in the details of structural recognition, the dynamic interplay between biomolecules is precisely orchestrated by the thermodynamics of the biomolecular equilibria involved. A consequence is that biochemists of all persuasions are drawn into the quantitative characterization of biomolecular interactions.

Fortunately, a common set of physicochemical principles applies to all such phenomena, irrespective of whether the interaction of interest involves an enzyme and its substrate or inhibitor, a hormone or growth factor and its receptor, an antibody and its antigen, or, indeed, the binding of effector molecules that modulate these interactions. The binding affinity, binding specificity, number of binding sites per molecule, as well as the enthalpic and entropic contributions to the binding energy are common parameters that assist an understanding of the biochemical outcome. This unit aims to provide an overview of the design and interpretation of binding experiments. More extensive treatments are provided by two recent monographs (Winzor and Sawyer, 1995; Klotz, 1997).

RECTANGULAR HYPERBOLIC BINDING RESPONSES

In studies of reversible interactions between two species, the smaller solute is termed the ligand (S) and is usually considered to possess a single site for interaction with the larger species. The latter, designated here as A, is termed either the acceptor or the receptor (if particulate), which may possess several sites for interaction with ligand. For a system entailing the binding of a ligand to p sites on an acceptor, the concentrations of p complexes ($AS, AS_2, .., AS_p$) as well as those of free acceptor and free ligand are required, in principle, for complete description of the composition of an acceptor-ligand mixture. However, under favorable circumstances the problem simplifies to quantitative characterization by determining the free concentration of ligand (C_S) in a mixture with defined total concentrations of acceptor and ligand ($\overline{C}_A$ and $\overline{C}_S$, respectively). This simplification arises when the concentration of bound ligand ($\overline{C}_S - C_S$) exhibits a rectangular hyperbolic dependence upon free ligand concentration (C_S) for mixtures with a fixed total acceptor concentration ($\overline{C}_A$). Such a situation is encountered, for example, with enzymes exhibiting Michaelis-Menten behavior, where the observed dependence of initial velocity upon substrate concentration is the manifestation of a rectangular hyperbolic binding response.

Although binding theory is usually derived for the general case in which a ligand binds to p sites on an acceptor, this discussion presents an alternative derivation for the simpler case where ligand binding is restricted to two acceptor sites. For this system, the various equilibria are described in terms of molar association constants (K_i) by:

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$$-\mathrm{A}-+\mathrm{S} \overset{K_1}{\rightleftharpoons} -\mathrm{A}-\mathrm{S} \quad C_{\mathrm{AS}} = K_1 C_{\mathrm{A}} C_{\mathrm{S}}$$

Equation A.5A.1

$$-\mathrm{A}-+\mathrm{S} \overset{K_2}{\rightleftharpoons} \mathrm{S}-\mathrm{A}- \quad C_{\mathrm{SA}} = K_2 C_{\mathrm{A}} C_{\mathrm{S}}$$

Equation A.5A.2

$$-\mathrm{A}-\mathrm{S}+\mathrm{S} \overset{K_3}{\rightleftharpoons} \mathrm{S}-A-\mathrm{S} \quad C_{\mathrm{SAS}} = K_3 C_{\mathrm{AS}} C_{\mathrm{S}} = K_1 K_3 C_{\mathrm{A}} C_{\mathrm{S}}^2$$

Equation A.5A.3

$$\mathrm{S}-\mathrm{A}-+\mathrm{S} \overset{K_4}{\rightleftharpoons} \mathrm{S}-A-\mathrm{S} \quad C_{\mathrm{SAS}} = K_4 C_{\mathrm{SA}} C_{\mathrm{S}} = K_2 K_4 C_{\mathrm{A}} C_{\mathrm{S}}^2$$

Equation A.5A.4

Completely general characterization thus requires the determination of four equilibrium constants, the only interrelationship between which is identity of the products K_1K_3 and K_2K_4 to maintain independence of the concentration of saturated acceptor-ligand complex (C_{SAS}) upon the pathway of its formation—a thermodynamic necessity because the concentration of saturated complex is either $K_1K_3C_{\mathrm{A}}C_{\mathrm{S}}^2$ or $K_2K_4C_{\mathrm{A}}C_{\mathrm{S}}^2$. The total concentrations of acceptor and ligand may therefore be written in the form:

$$\overline{C}_{\mathrm{A}} = C_{\mathrm{A}}\left[1+(K_1+K_2)C_{\mathrm{S}} + K_1K_3C_{\mathrm{S}}^2\right]$$

Equation A.5A.5

$$\overline{C}_{\mathrm{S}} = C_{\mathrm{S}} + C_{\mathrm{A}}\left[(K_1+K_2)C_{\mathrm{S}} + 2K_1K_3C_{\mathrm{S}}^2\right]$$

Equation A.5A.6

In an experimental context the binding response is usually expressed in terms of a binding function, termed r (Klotz, 1946) or ν (Scatchard, 1949) and defined as the molar ratio of the amount of bound ligand to the total amount of acceptor. On the grounds that these two amounts are contained in the same volume, the binding function for the above system is given by Equation A.5A.7, which is the binding equation in Adair (1925) format.

$$r = \left(\overline{C}_{\mathrm{S}} - C_{\mathrm{S}}\right) / \overline{C}_{\mathrm{A}} = \frac{(K_1+K_2)C_{\mathrm{S}} + 2K_1K_3C_{\mathrm{S}}^2}{1+(K_1+K_2)C_{\mathrm{S}} + K_1K_3C_{\mathrm{S}}^2}$$

Equation A.5A.7

Simplification of Equation A.5A.7 to a rectangular hyperbolic dependence of the binding function upon free ligand concentration entails the incorporation of two separate assumptions: (1) binding sites on the acceptor are equivalent ($K_1 = K_2$), and (2) binding sites on the acceptor are independent ($K_1 = K_4$, $K_2 = K_3$). The former implies identity of the binding characteristics for the interaction of ligand with either site on (for example) a dimeric enzyme comprising two identical subunits, whereas the latter assumption implies that the same binding constant applies to ligand interaction with a given site, irrespective of the occupancy state of the other. These two assumptions allow the description of binding in

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terms of a rectangular hyperbolic response governed by a single intrinsic (site-binding) constant, $K_{AS} = K_1 = K_2 = K_3 = K_4$. With these substitutions, Equation A.5A.7 becomes:

$$r = \frac{2K_{AS}C_S}{1 + K_{AS}C_S}$$

Equation A.5A.8

which is the particular form ($p = 2$) of the general expression for a rectangular hyperbolic binding response, namely:

$$r = \frac{pK_{AS}C_S}{1 + K_{AS}C_S}$$

Equation A.5A.9

where p denotes the number of equivalent and independent ligand-binding sites on the acceptor. Because there is also widespread use of the definition of equilibrium constants in terms of dissociation ($K_d = 1/K_{AS}$), Equation A.5A.9 can also be written in the form:

$$r = \frac{pC_S}{K_d + C_S}$$

Equation A.5A.10

which bears obvious analogies to the Michaelis-Menten equation.

The general derivation of Equation A.5A.9 (e.g., Klotz, 1946; Winzor and Sawyer, 1995) is certainly more elegant than that presented here, but its reliance upon statistical considerations dictates the introduction of site equivalence and site independence as a combined assumption. The present treatment emphasizes that there are two separate assumptions, either of which may not be fulfilled. For example, binding sites may be independent but nonidentical—the situation encountered in the binding of protons to the amino and carboxyl groups of glycine. In addition to the possibility of acceptor-site heterogeneity, there may be sites that are equivalent ($K_1 = K_2$) but dependent ($K_1 \neq K_4$, $K_2 \neq K_3$)—the basis of a common concept of allostery (Koshland et al., 1966). However, violation of either assumption leads to binding curves that deviate from rectangular hyperbolic form. In Figure A.5A.1, the solid curve depicts the dependence of r upon C_S under conditions where both assumptions prevail, whereas the broken lines correspond to examples of acceptor site heterogeneity (- - -) and acceptor site cooperativity with $K_4 > K_1$ (– – –).

Rectangular hyperbolic responses abound in biology; in many cases the source can be traced to a single equilibrium binding event. There are situations where the parameter being monitored is the actual binding process. On the other hand, the kinetic characterization of an enzyme exhibiting Michaelis-Menten kinetic behavior provides an example where the rectangular hyperbolic dependence of the initial velocity (v) of product formation reflects the interactions of substrate with enzyme and the various enzyme-substrate complexes (ES, ES_2,.., ES_{p-1}). In present terms, the maximal velocity, V, is given by the product of the catalytic rate constant (k_c) and the total enzyme concentration ($\overline{C}_A$), whereupon the initial velocity is described by the relationship

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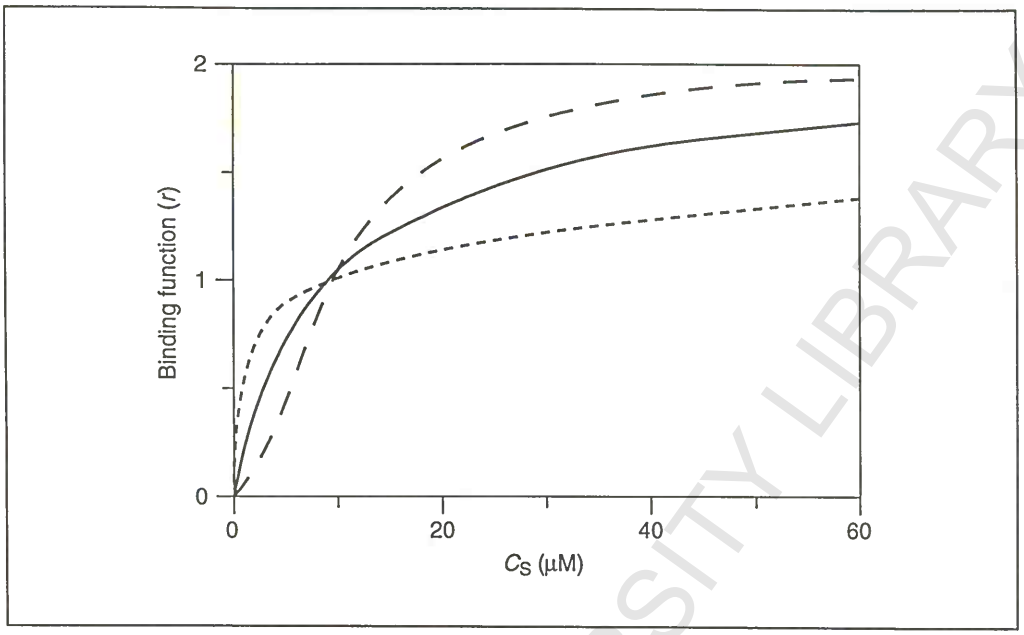

Figure A.5A.1 Illustrative dependences of the binding function (r) upon free ligand concentration (C_S) for various types of acceptor-ligand interaction. Rectangular hyperbolic dependence (——) for an acceptor with two equivalent and independent sites ($K_{AS} = K_1 = K_2 = K_3 = K_4 = 10^5\ M^{-1}$). Binding curve (- - -) for an acceptor with two independent but nonequivalent sites ($K_1 = K_4 = 10^6\ M^{-1}$; $K_2 = K_3 = 10^4\ M^{-1}$). Sigmoidal binding curve (– – –) for an acceptor with two equivalent but dependent sites ($K_1 = K_2 = 10^4\ M^{-1}$; $K_3 = K_4 = 10^6\ M^{-1}$). See Equations A.5A.1-A.5A.4 for equilibrium constant nomenclature.

$$v = k_c r \overline{C}_A = \frac{p k_c \overline{C}_A C_S}{K_M + C_S}$$

Equation A.5A.11

This emphasizes the fact that the catalytic rate constant and the Michaelis constant (K_M) are intrinsic parameters obtained on the basis of equivalent and independent substrate binding to the p active sites on the enzyme.

QUANTITATIVE CHARACTERIZATION OF A HYPERBOLIC RESPONSE

Quantitative characterization of a rectangular hyperbolic binding response entails the determination of two parameters, p and K_{AS}, in Equation A.5A.9. In the design of a binding experiment it is therefore important to adopt an experimental strategy that will maximize the accuracy with which those two parameters may be estimated from the measured dependence of the binding function (r) upon free ligand concentration (C_S).

Importance of Studying a Wide Range of Ligand Concentrations

The desired starting point in any binding analysis is a well-defined dependence of the binding function upon C_S. Under the equilibrium conditions of a binding assay, the magnitude of r approaches its maximum limiting value (p) very gradually with increasing free ligand concentration (Fig. A.5A.1). A wide range of C_S values is therefore required to extract accurate values of both p and K_{AS} from the form of the equilibrium binding curve. Whereas a linear scale in free ligand concentration crowds data at low C_S values because of substantial changes in r for small changes in free ligand concentration, a logarithmic scale in C_S turns the plot into a sigmoidal curve that is symmetrical about an

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inflection point at $r = p/2$. For rectangular hyperbolic binding, this inflection point provides an estimate of $K_d = 1/K_{AS}$. A change by one log unit (base 10) of C_S on each side of this inflection point covers a range of 10% to 90% acceptor saturation. Consequently, the dependence of r upon $\log_{10}C_S$ is an extremely useful means of assessing whether a sufficient range in r has been covered in the binding assay to allow accurate extraction of values of p and K_{AS} (Thompson and Klotz, 1971). The restriction of measurements to a narrow range of C_S, and hence of r, also renders difficult the detection of any deviation from rectangular hyperbolic behavior.

In situations where either the solubility or insufficiency of ligand precludes measurements of the upper portion of the binding curve, advantage may sometimes be taken of a change to experimental conditions that ensure essentially stoichiometric binding of ligand to acceptor. Such selection of conditions where the binding constant is sufficiently great for the acceptor-ligand mixtures to comprise complex and excess reactant (acceptor or ligand), the binding function (or a parameter related thereto) becomes linear in total ligand concentration to the titration endpoint, and independent of ligand concentration thereafter (Fig. A.5A.2A). Unequivocal determination of the reaction stoichiometry by this means or by use of subsidiary information, such as knowledge of the quaternary state of the acceptor, simplifies analysis of the binding data under equilibrium conditions (Fig. A.5A.2B) to the determination of a single parameter (K_{AS}), and hence enhances the accuracy with which the binding constant may be obtained from a suboptimal set of experimental results.

Nonlinear Least-Squares Methods

Direct fitting of untransformed data to the appropriate binding expression is the preferred method of evaluating binding parameters. Experimental binding data comprising (r, C_s) coordinate pairs can be fitted directly to Equation A.5A.9 by any of a number of nonlinear least-squares curve-fitting programs that are available in commercial software packages. Good programs allow the fitting equation to be defined by the user, and a choice of the parameters to be floated during the fitting procedure. The program should also provide estimates of the errors in the fitted parameters, as well as assessments of the goodness-of-fit in terms of chi-squared values and plots of the residuals. The first point on which the experimenter must decide is whether the data are indicative of a rectangular hyperbolic function. An objective test of the goodness-of-fit is provided by the value of chi squared. Visual inspection of the residuals plot should reveal a random scatter of experimental data about the fitted curve. Clearly, these tests require that there be sufficient data points or a sufficient sample size for statistical tests to be meaningful. For practical reasons this requirement is not met in many binding assays. However, for many spectroscopic titrations there is no impediment to collecting the required number of data points.

The experimenter should also be aware of the limitations of the fitting procedure. Most programs are based on a modified Marquardt algorithm, which may have difficulty converging to a minimum chi squared when initial estimates of the parameters are far removed from those associated with the least squares minimum. Moreover, the experimental points must cover a sufficient range of acceptor saturation. For example, programs experience difficulty returning accurate values of p and K_{AS} when experimental data are only available for a narrow range of acceptor saturation. Knowledge of the error associated with the estimates of both r and C_S is also important. Comparison of experimental results with simulated data for various values of K_{AS} (and possibly p) can be very useful for demonstrating the uniqueness of the fit obtained.

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Use of Linear Transforms

Before the general availability of computers, linear transforms of the rectangular hyperbolic dependence of r upon C_S were used to determine values of p and K_{AS}. By simple algebraic manipulation, Equation A.5A.9 can be transformed into the following expressions:

$$r/C_S = pK_{AS} - rK_{AS}$$

Equation A.5A.12

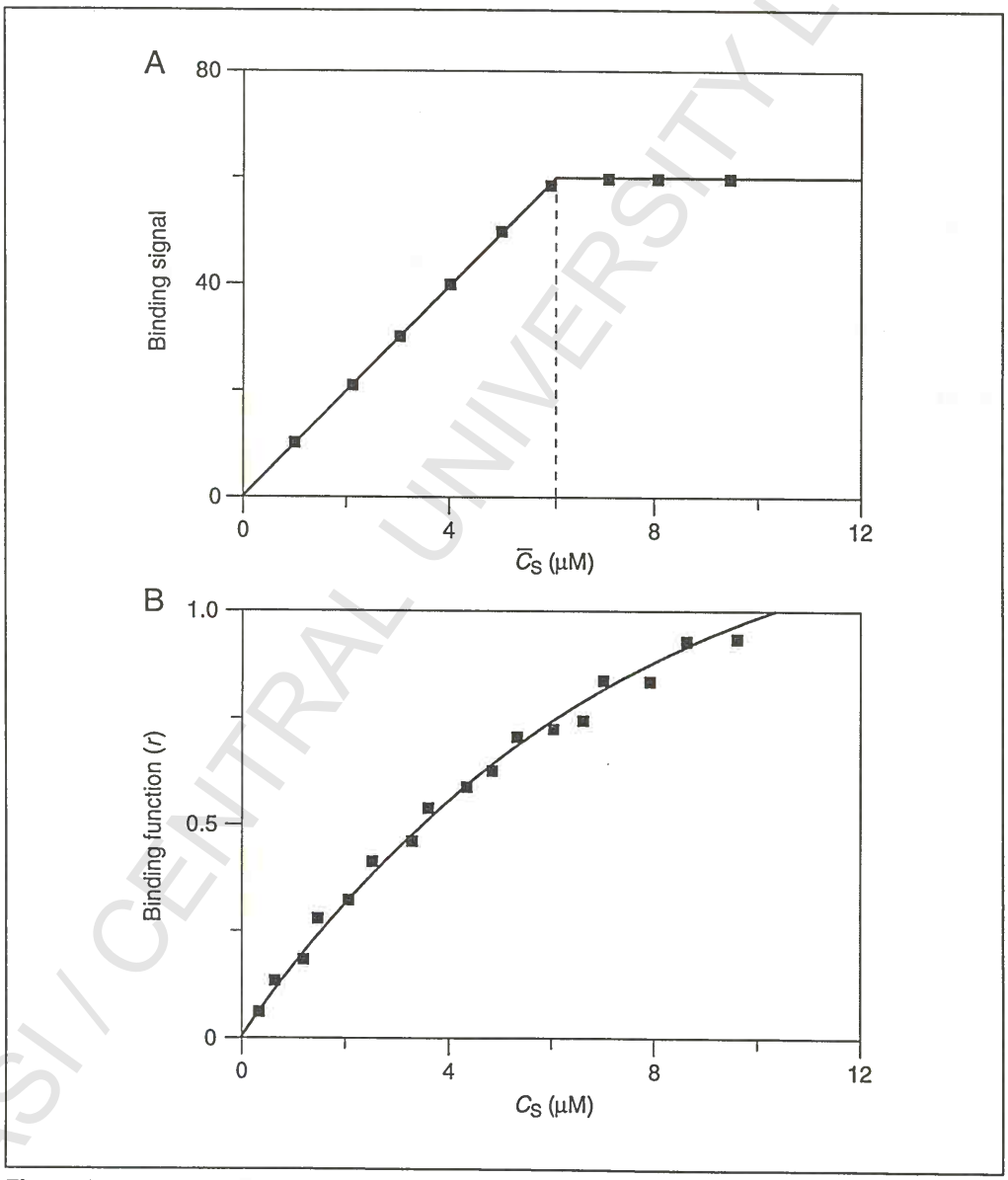

Figure A.5A.2 Illustrative characterization of an acceptor-ligand interaction for a system with limited ligand solubility. (**A**) Estimation of the number of binding sites by titration of 3 μM acceptor with ligand under stoichiometric conditions: $p = 2$ on the grounds that 6 μM ligand is required to titrate 3 μM acceptor. (**B**) Binding data obtained (for example) by equilibrium dialysis over the limited range of ligand solubility (<10 μM), together with the best-fit rectangular hyperbolic relationship (Equation A.5A.9) for a system with $p = 2$ (see A).

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$$C_S/r = 1/(pK_{AS}) + C_S/p$$

Equation A.5A.13

$$1/r = 1/(pK_{AS}C_S) + 1/p$$

Equation A.5A.14

which lead to the graphical representations of data shown in Figure A.5A.3 (Scatchard plot, Hames plot, and double-reciprocal plot, respectively).

An undesirable characteristic of these plots is the bias introduced by the linear transformation. In the example given in Figure A.5A.3, the data become crowded towards the ordinate in the Hames and double-recip-

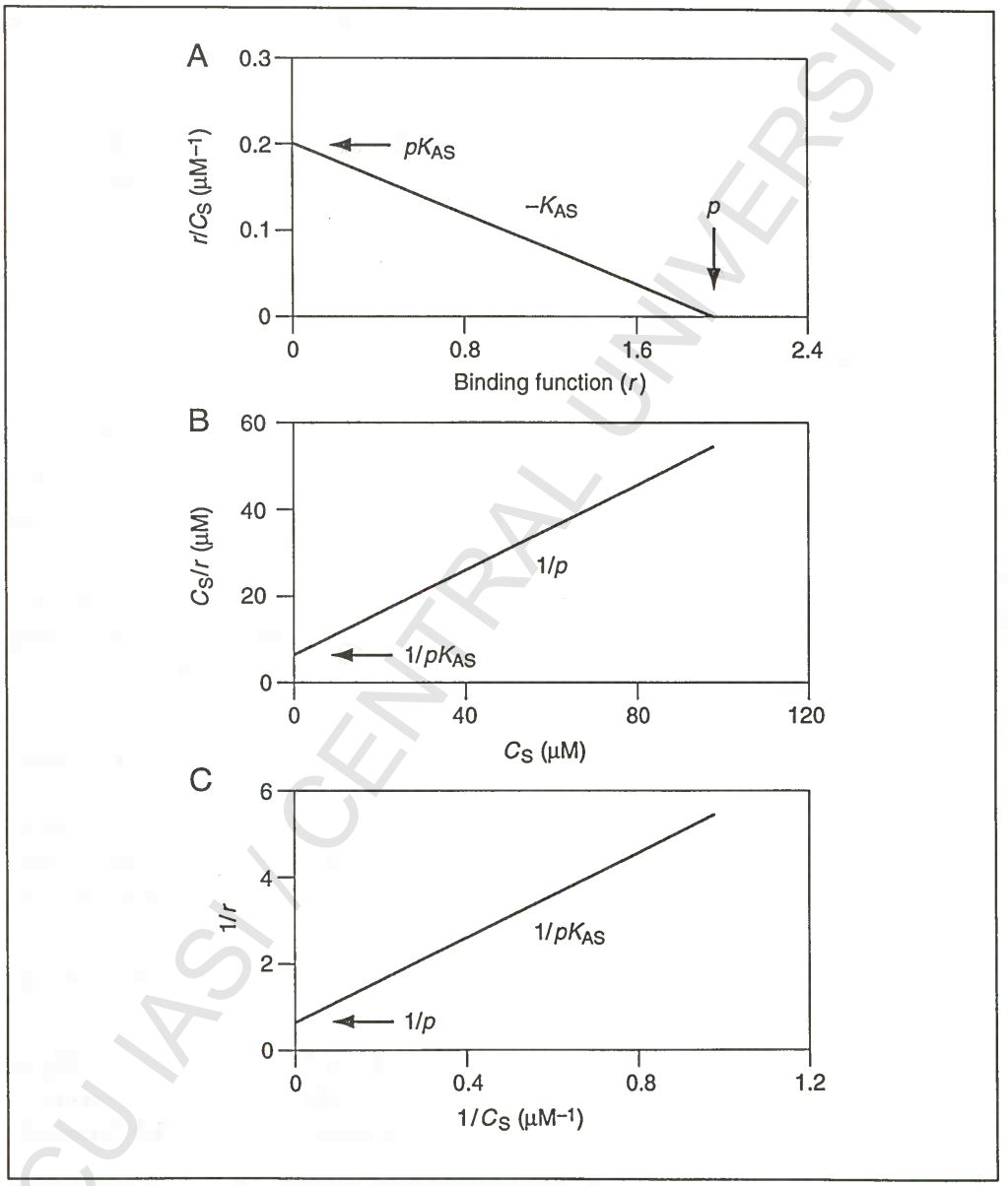

Figure A.5A.3 Use of various linear transforms (Equations A.5A.12-A.5A.14) of the rectangular hyperbolic binding equation to characterize an acceptor-ligand interaction with $p = 2$ and $K_{AS} = 10^5$ M^{-1}. (**A**) Scatchard plot; (**B**) Hames plot; (**C**) double-reciprocal plot.

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rocal plots. This distortion of the primary data results in variable weighing of points in a linear regression analysis, a point addressed by Klotz (1983, 1997). The nonlinear least-squares fitting procedure circumvents this problem.

Despite the superiority of nonlinear least-squares fitting procedures for determining binding parameters, the plot of binding data in accordance with one of these linear transforms can still serve an important role in the analysis. First, the linearly transformed plot can assist in the decision about the adequacy of the simplest model with binding to equivalent and independent acceptor sites. More complex modes of binding give rise to deviations from rectagular hyperbolic responses (Fig. A.5A.1), and hence from linearity in the transformed manner of presentation. It is easier for the human eye to detect deviations from linearity than subtle deviations from a curvilinear dependence. Second, the linear transform is also useful for providing initial estimates of p and K_{AS} that are required by nonlinear least-squares fitting programs.

Experimental design summary

1. Use a semilogarithmic plot of r versus $\log_{10}C_S$ to establish the adequacy of the range of acceptor saturation covered by the experimental measurements (preferably 10% to 90%).
2. Know the limitations of the experimental method used for determining binding data. Determine the respective (r, C_S) coordinate pairs at least in triplicate to allow assessment of the uncertainty associated with each value of r.
3. Collect as many (r, C_S) coordinate pairs as practicable to allow the effective use of residuals plots and the statistical evaluation of chi squared.

Data analysis summary

1. Analysis of binding data aims, in the first instance, to identify and characterize the most appropriate binding model that is consistent with the experimental data.
2. The simplest model involves the binding of a ligand to equivalent and independent sites on a single acceptor, and results in the rectangular hyperbolic behavior described above. It is therefore the first model to be tested.
3. Deviations from rectangular hyperbolic behavior, detected by unsatisfactory curve fitting of the untransformed or transformed data to the simplest situation, indicate that more complex binding models must be considered (see Binding Responses Deviating from Rectangular Hyperbolic Form).
4. The formulation of a new binding model, together with its mathematical description, frequently requires consideration of complementary information about the acceptor-ligand system: whether the acceptor undergoes isomerization or self-association, whether there are noninteracting forms of the acceptor, whether the ligand is multivalent, and whether binding is enhanced or inhibited by other effector molecules that may be present.
5. Simulation of the mathematical model can be useful in guiding the design of further experiments.

The process of data analysis and interpretation is depicted diagrammatically in Figure A.5A.4. In that regard, it must be appreciated that agreement of the model with the experimental data does not establish the mechanistic legitimacy of the model, but merely the consistency between the two.

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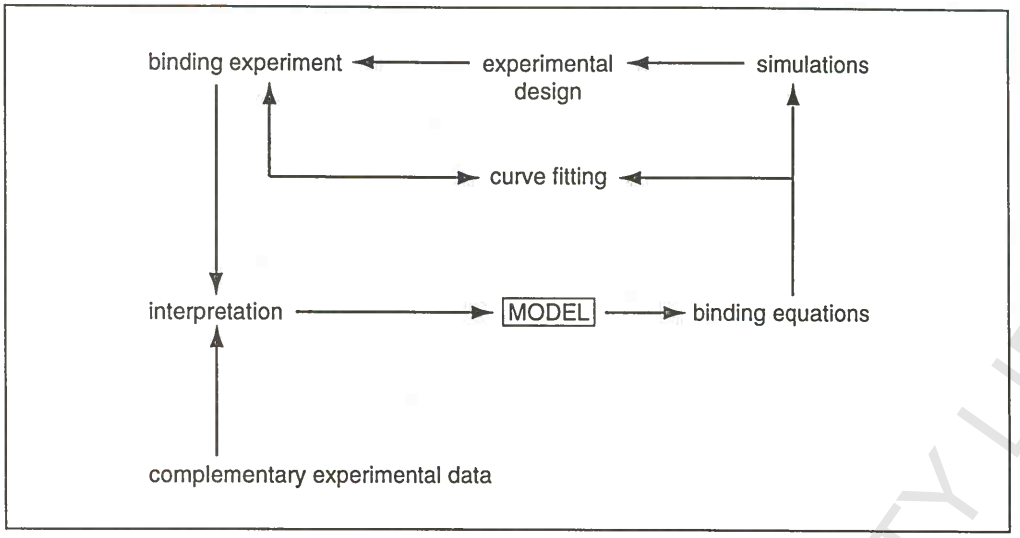

Figure A.5A.4 Schematic representation of the strategy for the quantitative characterization of a binding response. Taken from Winzor and Sawyer (1995).

ILLUSTRATIVE ANALYSES OF EXPERIMENTAL RESULTS

The quantitative characterization of ligand binding is usually envisaged in terms of procedures based on measurement of the free ligand concentration (C_S) in mixtures with defined total composition ($\overline{C}_A$, $\overline{C}_S$). However, spectral studies afford the opportunity to measure the concentration of complexed ligand ($\overline{C}_S - C_S$), in mixtures with defined total composition. Inasmuch as the concentrations of free and complexed ligand are both required for quantitative analysis of ligand binding, there is no theoretical advantage associated with either type of procedure.

Methods Based on Measurement of Free Ligand Concentration

In methods such as equilibrium dialysis (Klotz, 1946), steady-state dialysis (Colowick and Womack, 1969; Ford et al., 1984), diafiltration (Blatt et al., 1968), and ultrafiltration (Sophianopoulos et al., 1978), a membrane that is permeable to ligand but not to acceptor is used to create a two-phase system in which one phase comprises the equilibrium mixture with composition ($\overline{C}_A$, $\overline{C}_S$) and the other an acceptor-free solution of ligand at a concentration related directly to its equilibrium concentration (C_S). Other methods that yield equivalent information include frontal gel chromatography on a matrix that excludes acceptor and hence all acceptor-ligand complexes (Nichol and Winzor, 1964), and liquid-liquid partition studies (Goodman, 1958). Provided that the ligand is sufficiently small that complex formation has no effect on the migration rate of the acceptor, analytical ultracentrifugation also has the potential to generate a sedimentation velocity pattern in which the slower-migrating boundary comprises free ligand at its equilibrium concentration (Steinberg and Schachman, 1966).

For the first illustration of the quantitative characterization of ligand binding by measurement of the free ligand concentration in mixtures with known total composition, consider the often-studied interaction between methyl orange and bovine serum albumin. The semilogarithmic plot (Fig. A.5A.5A) summarizes the original equilibrium dialysis data (Klotz et al., 1946) as well as subsequent results obtained by sedimentation velocity (Steinberg and Schachman, 1966). The inset to Figure A.5A.5A shows the double-reciprocal transform of the equilibrium dialysis data that was used to deduce values of 22 and 2,200 M^{-1} for p and K_{AS}, respectively (Klotz et al., 1946). Nonlinear least-squares analysis of the combined data sets (Fig. A.5A.5B) in terms of Equation A.5A.9 yields

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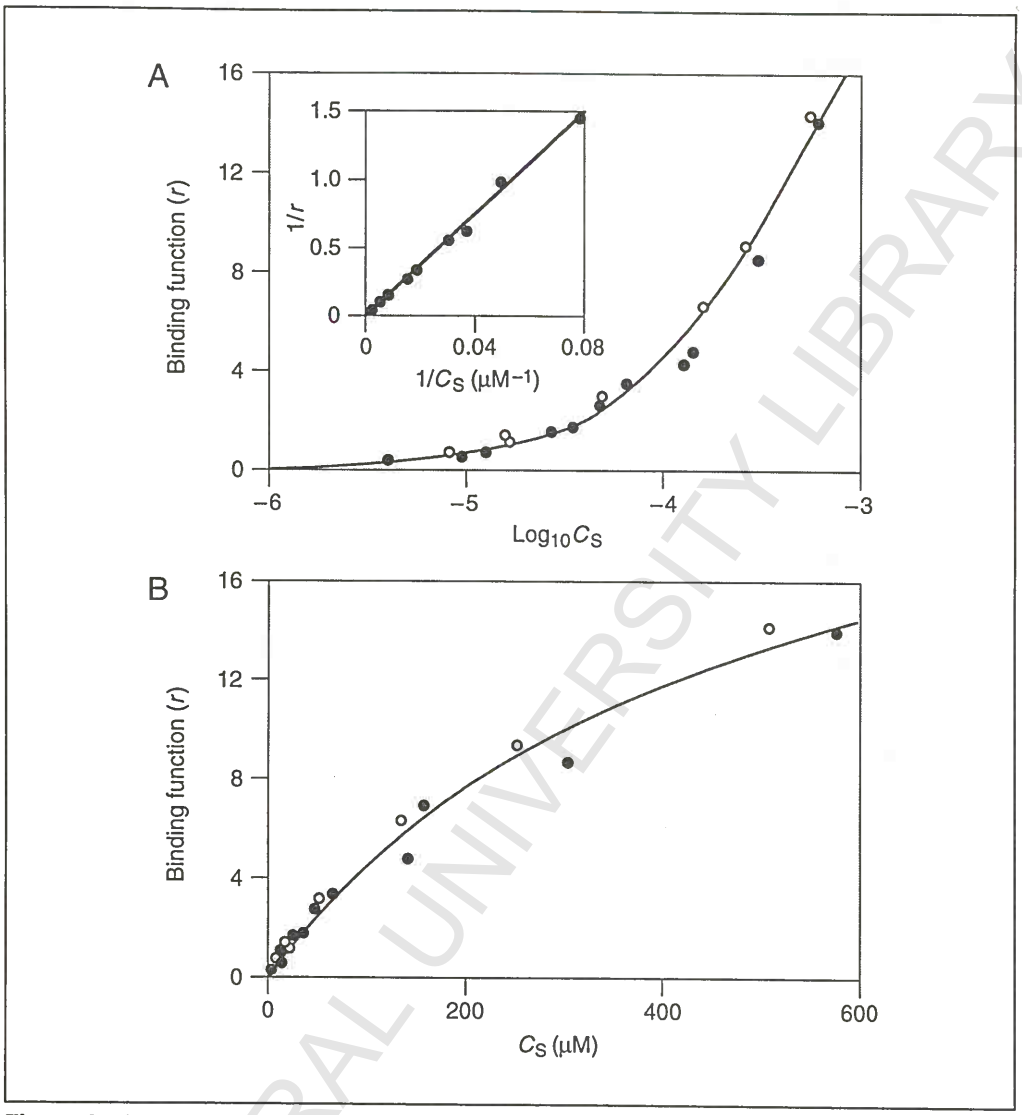

Figure A.5A.5 Results obtained by equilibrium dialysis (filled circles) and sedimentation velocity (open circles) for the interaction of methyl orange (S) with bovine serum albumin (A). (**A**) Semilogarithmic representation of the results, together with the double-reciprocal transform of the equilibrium dialysis data (inset). (**B**) Untransformed dependence of the binding function upon free ligand concentration, together with the best-fit description by nonlinear regression analysis according to Equation A.5A.9. Equilibrium dialysis data are taken from Klotz et al. (1946), and sedimentation velocity data from Steinberg and Schachman (1966).

respective estimates (at ±2 SD) of 27 ± 6 and 1900 ± 700 M^{-1} for the number of sites and the intrinsic binding constant. The relatively large extent of uncertainty associated with this quantitative analysis reflects the limited range of acceptor saturation (0.7% to 52%) covered by the binding measurements. Indeed, this deficiency of the data set is already manifested in the semilogarithmic plot, which shows no sign of the sigmoidal shape that indicates the coverage of a sufficient range of acceptor saturation for accurate definition of the affinity and stoichiometry of the interaction.

A second system is therefore used to better illustrate the potential of nonlinear least-squares analysis of binding data. The results of a frontal gel chromatographic study of the interaction between NADH and rabbit muscle lactate dehydrogenase (Ward and Winzor, 1983) have been supplemented with additional data to obtain the binding curve shown in Figure A.5A.6, which also establishes the sigmoidal shape of their representation in

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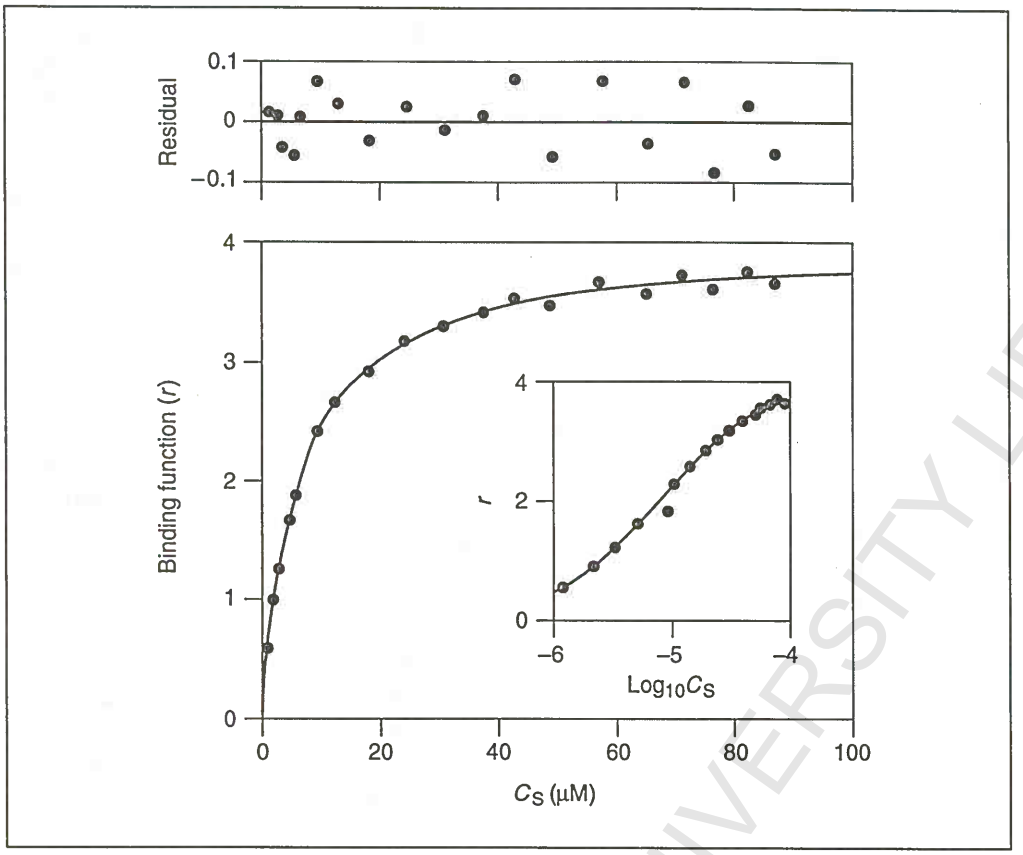

Figure A.5A.6 Characterization of the interaction between NADH and rabbit muscle lactate dehydrogenase (pH 7.4; ionic strength, *I*, 0.15) by nonlinear regression analysis of the untransformed gel chromatographic binding data in terms of Equation A.5A.9: the upper pattern shows random scatter in the residuals plot. The inset displays the same data in semilogarithmic format. Data taken in part from Ward and Winzor (1983).

semilogarithmic format (inset). The lines indicate the best-fit description obtained by nonlinear least-squares analysis, where $p = 4.01 \pm 0.04$ and $K_{AS} = 1.49\ (\pm 0.08) \times 10^5\ M^{-1}$, about which the experimental points exhibit random scatter (residuals plot in the upper panel). Inasmuch as the range of acceptor saturation ranges between 14.5% and 92.5%, these results exemplify the recommended experimental and analytical protocols that concluded the previous section.

Methods Based on Measurement of Complexed Ligand or Acceptor

Emphasis is now turned to binding procedures that rely upon the direct measurement of complex formation rather than on its deduction from knowledge of the total and free ligand concentrations. Because the extent of complex formation can be monitored by a response that emanates from either the acceptor or the ligand, there are two situations to consider.

A spectral signal arising from the acceptor

There are many examples in which a property of an acceptor is perturbed upon binding of a ligand. The property may be a biological activity, as in the case of an enzyme binding a substrate, or a biological response triggered by the occupation of a cell surface receptor. More commonly, a spectral property of the acceptor is changed upon ligand binding, thus providing a convenient method for following the binding reaction when the ligand is titrated into a solution of acceptor. The spectral change in the acceptor is a measure of the concentration of the ligand-acceptor complex ($\overline{C}_S - C_S$). Thus, the concentration of free

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ligand must be found by difference between the total ligand concentration ($\overline{C}_s$) and that which is bound, ($\overline{C}_S - C_S$). Besides providing a means of following the binding equilibrium, the spectral change may also provide information about the chemical groups in the acceptor that are located at the acceptor-ligand interface.

The use of spectroscopic techniques to monitor binding equilibria requires careful attention to spectroscopic purity. Ideally, the spectroscopic signal should arise only from the acceptor species, so that the signal from the acceptor alone can be used to determine its concentration and ultimately the stoichiometry of the binding reaction. If the spectroscopic signal arises from two or more species, only one of which binds the ligand, the spectral change is overlaid on the background signal from the nonbinding species. The titration is still valid provided that the concentration of the binding form is accurately known. Particular problems arise when an acceptor is labeled with an extrinsic fluorescent probe. Labeling is rarely 100% efficient, and the labeled and unlabeled material may differ in their affinity for the ligand. Much time can be saved by separating the labeled from the unlabeled material before conducting binding experiments. Small amounts of background signal (e.g., solvent fluorescence or scattered excitation light in the case of fluorescence spectroscopy) can be dealt with by using appropriate controls and background subtraction.

The ultimate aim of biochemical study is to explain in vivo biochemistry at the molecular level. Consequently, the biochemist frequently proceeds from a controlled reductionist environment to a complex in vivo environment in which experimental rigor may be at least partially compromised. In these complex systems, high-resolution techniques such as nuclear magnetic resonance (NMR) and electron spin resonance (ESR) are particularly valuable, as they can discern a signal from a single acceptor or ligand within extremely heterogeneous solutions.

Assuming that each bound ligand causes an equivalent change in the spectroscopic signal, the fractional change in the spectroscopic parameter (f_A) is given by:

$$f_A = (y - y_f)/(y_b - y_f)$$

Equation A.5A.15

where y_b and y_f are the signals from the acceptor when sites are fully occupied (bound) and unoccupied (free), respectively. Conversion of the fractional saturation (f_A) to the binding function (r) requires knowledge of the number of binding sites on the acceptor (p), that is, the stoichiometry of the reaction. The stoichiometry is determined from a separate titration carried out at high acceptor levels such that each amount of ligand added is fully bound to the acceptor. This condition is accomplished when the acceptor concentration is ~100-fold greater than the dissociation constant ($K_{AS}\overline{C}_A = 100$), whereupon a plot of the fractional saturation against the ratio of total ligand to total acceptor ($\overline{C}_S/\overline{C}_A$) consists of two linear segments that intersect at the endpoint of the titration, thereby providing the stoichiometry (Fig. A.5A.7, line a). The condition $K_{AS}\overline{C}_A = 10$ provides a lower limit for discerning the two linear segments (Fig. A.5A.7, line b).

Once the stoichiometry has been determined, the binding curve can be evaluated from a titration in which the acceptor concentration is close to the dissociation constant for the binding equilibrium (Fig. A.5A.7, line c). The value of r is established at each value of C_S, whereupon the concentration of free ligand becomes:

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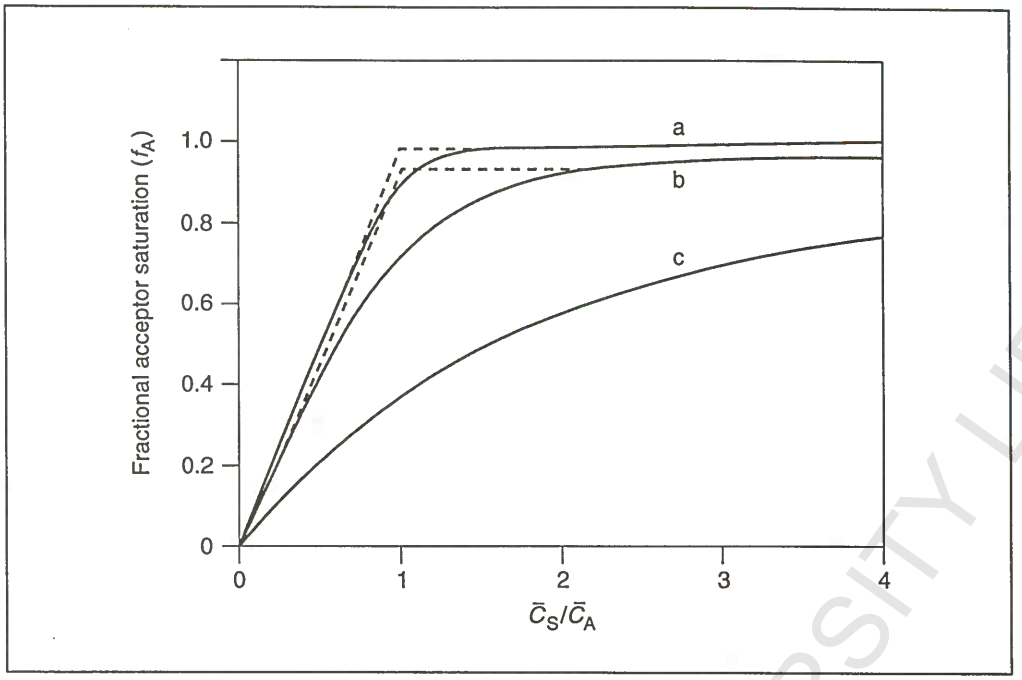

Figure A.5A.7 Evaluation of the stoichiometry of an acceptor-ligand interaction by stoichiometric titration. Curves have been constructed on the basis of a single acceptor site with a binding constant (K_{AS}) of 10^6 M^{-1} and total acceptor concentrations ($\bar{C}_A$) of 10^{-4} M (curve a), 10^{-5} M (curve b), and 10^{-6} M (curve c).

$$C_S = \bar{C}_S - r\bar{C}_A$$

Equation A.5A.16

The data are now plotted as r versus C_S or as one of the linear transforms described previously.

An example of this procedure is given by the binding of the *Escherichia coli* cAMP receptor protein to the primary site on the *lac* promoter (Heyduk and Lee, 1990). The stoichiometric titration in Figure A.5A.8A establishes the 1:1 stoichiometry, while the equilibrium titration in Figure A.5A.8B provides the data for generating the binding curve in Figure A.5A.8C. The binding analysis therefore requires two titrations—one under stoichiometric conditions, the other under equilibrium conditions.

A spectral signal arising from the ligand

Spectral properties of the ligand may be perturbed when it becomes bound to an acceptor. For example, changes may occur in the absorption or emission maximum, extinction coefficient, or circular dichroism ellipticity. The first step in binding analysis is determination of the spectral characteristics of the free and bound ligand. The characteristics of the free ligand (y_f) can be determined by direct measurement. Those of the bound ligand (y_b) can be determined by a separate titration in which acceptor is added to a solution of ligand. In the presence of excess acceptor all the ligand is bound. Plotting the data in double reciprocal form ($1/y$ versus $1/C_A$) allows the spectral property of the bound ligand to be discerned from the ordinate intercept. This plot is frequently assumed to be linear; however, examination of the theory shows that this need not be the case (Haigh and Sawyer, 1978). In the case of multiple equivalent or nonequivalent binding sites, it is assumed that the binding of each ligand results in an equivalent spectral change. A second titration is now carried out under equilibrium conditions in which a ligand is added to a

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constant concentration of acceptor. The fraction of the ligand bound (f_S) may be found from the expression:

$$f_S = (y - y_f)/(y_b - y_f)$$

Equation A.5A.17

where y is the measured signal at a particular concentration of ligand. It then follows that

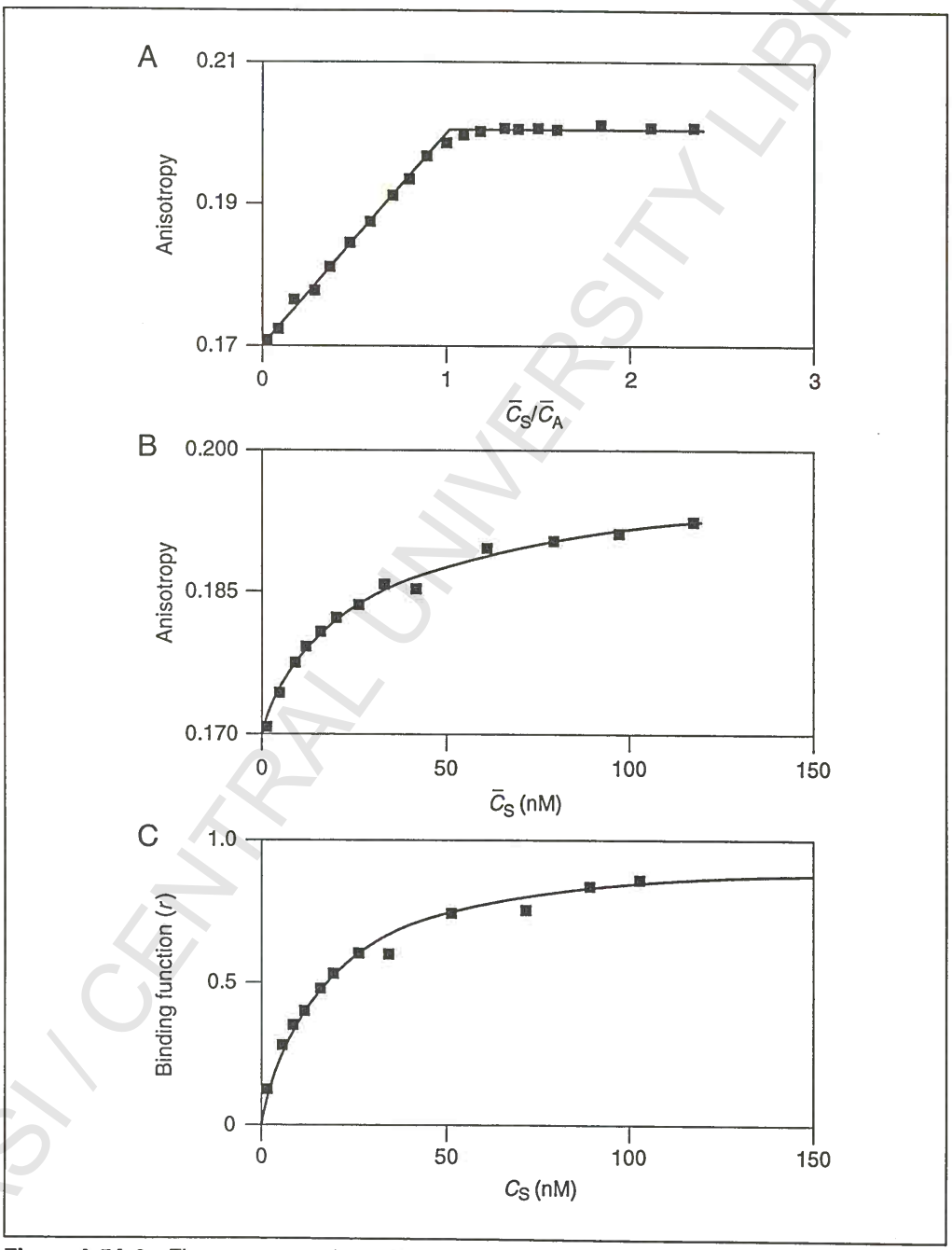

Figure A.5A.8 Fluorescence polarization (anisotropy) study of the interaction between *E. coli* cAMP receptor protein and a 32-bp DNA fragment of the *lac* promoter. (**A**) Stoichiometric titration of the fluorescently labeled DNA fragment with receptor protein in the presence of 0.2 mM cAMP. (**B**) Equilibrium titration in the presence of 0.5 μM cAMP. (**C**) Secondary plot of the equilibrium titration data as a binding curve, together with the best fit description ($p = 1$, $K_{AS} = 6.0 \times 10^7$ M^{-1}). Data taken from Heyduk and Lee (1990).

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$$r = f_S \overline{C}_S / \overline{C}_A$$

Equation A.5A.18

$$C_S = \overline{C}_S - f_S \overline{C}_S$$

Equation A.5A.19

These calculations are repeated at each concentration of ligand added, so that the single titration provides the entire binding curve.

Alternative approaches

Other procedures have been devised for the analysis of spectroscopic binding data. The dilution titration (Anderson and Weber, 1965) can be applied to situations where the spectroscopic signal originates from either the acceptor or the ligand, and to situations where dilution of the acceptor does not cause any dissociation of an acceptor protein oligomer. An iterative procedure can be applied to situations involving equivalent and independent sites (Holbrook, 1972; Holbrook et al., 1972; Shanley et al., 1985). A model independent procedure, which avoids the restrictive assumption that each ligand causes an equivalent change in the spectroscopic signal, has been devised by Chatelier and Sawyer (1987).

Competitive Binding Assays

In addition to methods in which an acceptor-ligand interaction is monitored directly, there are also procedures wherein equilibrium parameters for the interaction of interest are inferred from the inhibitory effect of a competing ligand on an acceptor interaction that has already been characterized. Such competitive binding assays abound in the screening of drug and antigen analogs by solid-phase radioimmunoassay and enzyme-linked immunosorbant assay (ELISA) techniques, and also in the characterization of interactions by quantitative affinity chromatography (Winzor, 1997) and biosensor technology (Hall and Winzor, 1998). The analysis of competitive binding assays has been considered most extensively in the context of quantitative affinity chromatography, and is therefore presented in that light.

General theoretical aspects

In solid-phase competitive binding assays, the matrix-associated reactant (X) is chosen to obtain selective interaction with the solute of interest (acceptor A). For example, the interaction of antithrombin (A) with heparin (S) has been studied (Hogg et al., 1991) using heparin immobilized on Sepharose as the matrix-bound reactant (X). Ligand-facilitated elution of acceptor (A) from the matrix thus occurs because its interactions with ligand (S, soluble heparin) and matrix sites (X, immobilized heparin) are mutually exclusive (Fig. A.5A.9).

The first step of a competitive binding assay entails determination of the binding constant for the acceptor-matrix interaction (K_{AX}) and an associated curve-fitting parameter, the effective total concentration of matrix sites ($\overline{\overline{C}}_X$). Provided that A is univalent in its interaction with matrix sites, the extent of acceptor depletion from the liquid phase exhibits a rectangular hyperbolic dependence upon the acceptor concentration in the liquid phase ($\overline{C}_A$). For a mixture in which the total acceptor concentration in the system is $\overline{\overline{C}}_A$, the rectangular hyperbolic dependence may be written in the form

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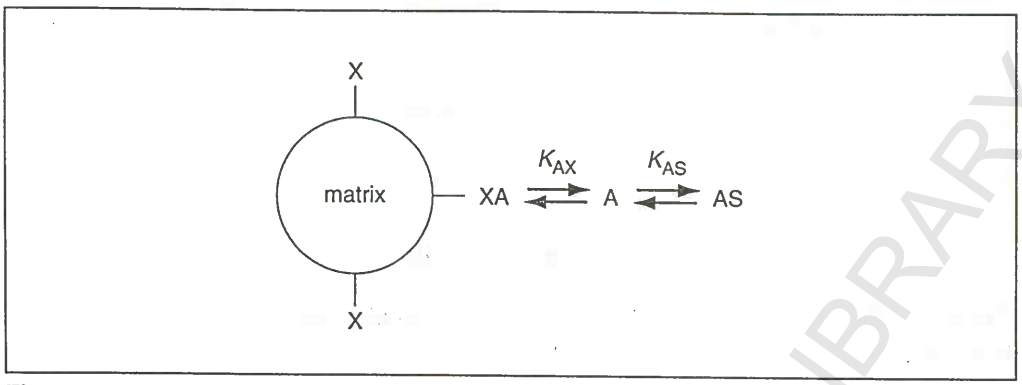

Figure A.5A.9 Schematic representation of the competitive interactions of soluble ligand (S) and immobilized affinity sites (X) for acceptor (A) in quantitative affinity chromatography.

$$\overline{\overline{C}}_A - \overline{C}_A = K_{AX}\,\overline{\overline{C}}_X\,\overline{C}_A/(1+K_{AX}\overline{C}_A)$$

Equation A.5A.20

of which the most convenient version of the Scatchard linear transform is

$$\left(\overline{\overline{C}}_A/\overline{C}_A\right)-1 = K_{AX}\overline{C}_X - K_{AX}\overline{C}_A\left[\left(\overline{\overline{C}}_A/\overline{C}_A\right)-1\right]$$

Equation A.5A.21

Expression of the Scatchard transform in that manner allows its ready adaptation to column chromatographic data, for which the ratio $\overline{\overline{C}}_A/\overline{C}_A$ may be replaced by $\overline{V}_A/V_A{}^*$, where $\overline{V}_A$ is the measured elution volume of acceptor on an affinity column for which $V_A{}^*$ is the corresponding elution volume in the absence of any acceptor-matrix interaction (the elution volume of ligand-saturated A). For frontal chromatographic studies, the counterpart of Equation A.5A.21 thus becomes (Hogg and Winzor, 1984)

$$\left(\overline{V}_A/V_A^*\right)-1 = K_{AX}\,\overline{\overline{C}}_X - K_{AX}\overline{C}_A\left[\left(\overline{V}_A/V_A^*\right)-1\right]$$

Equation A.5A.22

Upon supplementation of the liquid phase with a free concentration (C_S) of competing ligand, the rectangular hyperbolic dependence of ($\overline{\overline{C}}_A - \overline{C}_A$) upon $\overline{C}_A$ continues to be described by Equation A.5A.20, except that the acceptor-matrix binding constant so determined is a constitutive parameter ($\overline{K}_{AX}$) that is related to K_{AX} by the relationship (Winzor and Jackson, 1993)

$$Q = K_{AX}/\overline{K}_{AX} = 1 + K_{AS}C_S$$

Equation A.5A.23

The binding constant for the competing acceptor-ligand interaction in solution (K_{AS}) may therefore be obtained from the linear dependence of the ratio of acceptor-matrix binding constants (Q) upon free ligand concentration. In instances where the total ligand concentration ($\overline{C}_S$) is the parameter of known magnitude, Equation A.5A.23 is replaced by the expression

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$$Q = 1 + K_{AS}\left[q\overline{C}_S - (Q-1)\overline{C}_A/Q\right]$$

Equation A.5A.24

where q is the valence of S in its interaction with A, a consideration necessitated by the enforced design of the experiment whereby A must be univalent in its interaction with matrix sites X. The problem of allowing A to be multivalent is addressed later.

Partition equilibrium variants of quantitative affinity chromatography

The most direct method of employing Equation A.5A.20 or A.5A.21 is to perform simple partition equilibrium experiments on matrix-acceptor mixtures (with or without S). Measurement of the liquid-phase concentration of A after attainment of equilibrium allows the concentration of matrix-bound solute to be determined as the difference between the total acceptor concentration in the matrix slurry and its corresponding concentration in the liquid phase (Nichol et al., 1974). The problem of ensuring that each mixture contains the same amount of affinity matrix can frequently be overcome by using a recycling partition technique (Ford and Winzor, 1981; Hogg et al., 1991) in which the liquid-phase concentration of acceptor ($\overline{C}_A$) is monitored spectrophotometrically by means of a flow cell placed in the line returning the liquid phase to the slurry (Fig. A.5A.10A). This procedure has the advantage that a number of partition experiments may be performed with the same sample of matrix by making successive additions of concentrated acceptor solution to the slurry and determining the corresponding value of $\overline{C}_A$ after each addition. The dilution of the concentration of affinity sites ($\overline{\overline{C}}_X$) arising from the stepwise addition of acceptor (and ligand) aliquots with volume δV is accommodated by multiplying each ($\overline{\overline{C}}_A - \overline{C}_A$) result by $V_A^*/(V_A^*)_0$: the relevant volume accessible to A after n such additions (V_A^*) is calculated from the initial value $(V_A^*)_0$ and the relationship $V_A^* = (V_A^*)_0 + n\delta V$. A Scatchard representation of results obtained by this technique for the interaction of antithrombin with heparin-Sepharose (Hogg et al., 1991) is presented in Figure A.5A.10B.

Column chromatographic variants

Inspection of Equation A.5A.22 shows that knowledge of $\overline{C}_A$, the concentration of acceptor in the liquid phase, is a prerequisite for its application to elution profiles obtained by applying acceptor solution (or acceptor-ligand mixture) to an affinity column. However, there have been relatively few chromatographic studies in which the full potential of Equation A.5A.22 has been put to advantage (Winzor, 1997). Far greater use has been made of zonal chromatography, where the lack of a value of $\overline{C}_A$ dictates the truncation of Equation A.5A.22 to:

$$\left(\overline{V}_A/V_A^*\right) - 1 = K_{AX}\,\overline{\overline{C}}_X$$

Equation A.5A.25

which involves the assumption that the total concentration of matrix sites is an acceptable substitute for their free concentration (Bergman and Winzor, 1986). Subject to the validity of that approximation, zonal chromatography of the solute on an affinity column preequilibrated with a range of concentrations of competing ligand (C_S) allows the acceptor-ligand binding constant to be evaluated from the expression

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$$\left(\overline{V}_A/V_A^*\right)-1=K_{AX}\,\overline{\overline{C}}_X/\left(1+K_{AS}C_S\right)$$

Equation A.5A.26

in which K_{AS} and the product $K_{AX}\overline{\overline{C}}_X$ are the two parameters to be obtained by curve fitting. Further details of this procedure, introduced by Dunn and Chaiken (1974), are to be found in a recent review of quantitative affinity chromatography (Winzor, 1997).

Biosensor studies of ligand binding

An alternative means of quantifying the interaction between acceptor and matrix sites is to directly monitor the concentration of matrix-bound A in an equilibrium mixture by

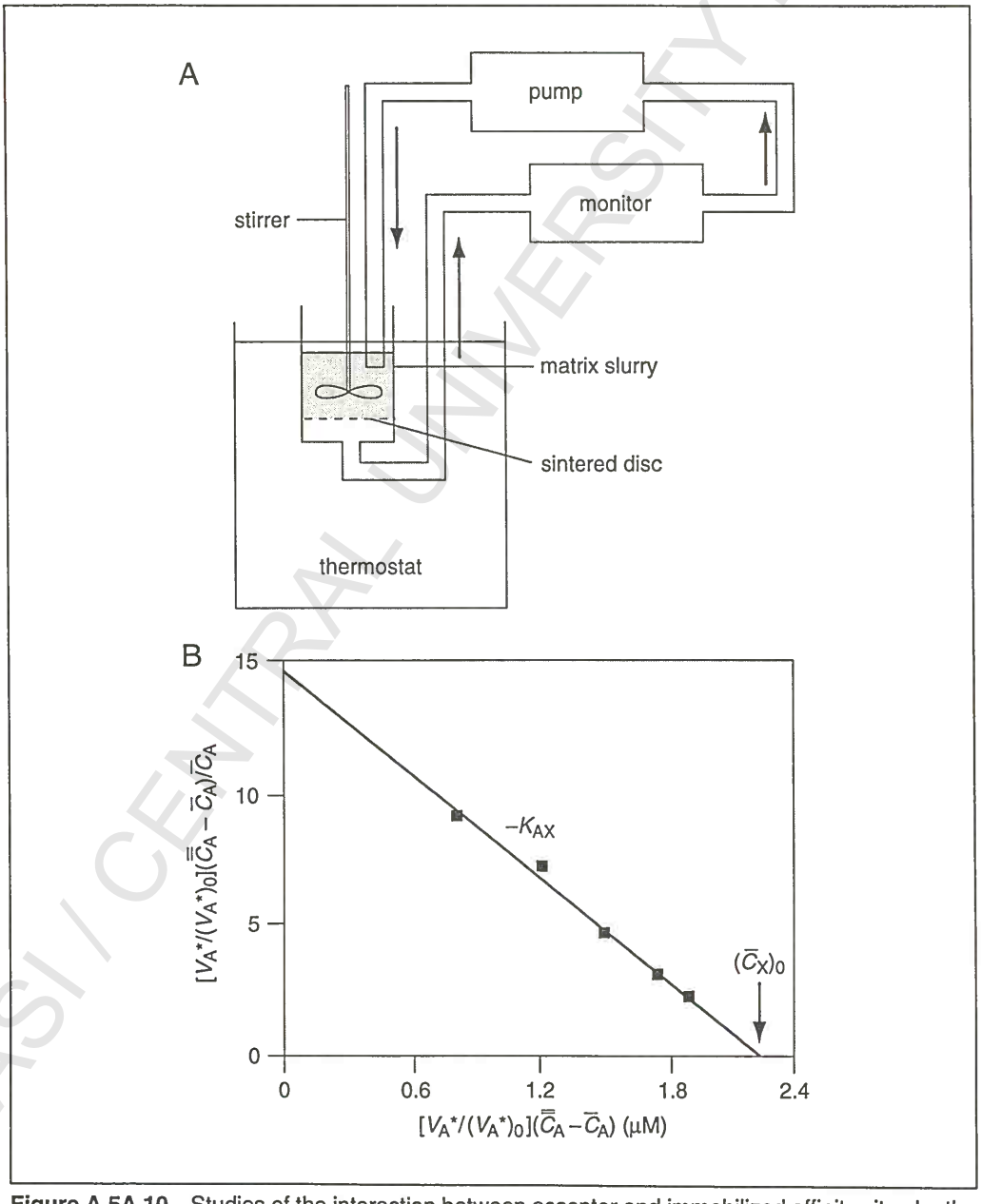

Figure A.5A.10 Studies of the interaction between acceptor and immobilized affinity sites by the recycling partition variant of quantitative affinity chromatography. (**A**) Schematic representation of the experimental system. (**B**) Scatchard plot of results obtained for the interaction of antithrombin with heparin-Sepharose (Hogg et al., 1991).

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biosensor technologies such as those incorporated into the BIAcore and IAsys instruments. Although the major interest in biosensor-based instruments has been their use to determine the kinetics of adsorption and desorption for the acceptor-matrix interaction, attention has also been drawn to the potential of their application in the context of quantitative affinity chromatography (Ward et al., 1995; Hall and Winzor, 1997, 1998).

In the BIAcore instrumentation, a solution of acceptor flows through a capillary channel over the biosensor chip, which has been rendered an affinity matrix by the covalent attachment of affinity sites X. Because the solution in contact with the sensor surface is being replaced continually by fresh solution, the time-independent response that is ultimately attained (R_e) is directly proportional to the concentration of bound acceptor ($\overline{\overline{C}}_A - \overline{C}_A$) in equilibrium with $\overline{C}_A$, the concentration of acceptor in the solution being injected into the instrument. Measurement of R_e as a function of injected acceptor concentration ($\overline{C}_A$) allows K_{AX} to be determined from the expression

$$R_e = K_{AX}\left(B\overline{\overline{C}}_X\right)\overline{C}_A/\left(1+K_{AX}\overline{C}_A\right)$$

Equation A.5A.27

which is essentially a rearranged form of Equation A.5A.20 with ($\overline{\overline{C}}_A - \overline{C}_A$) replaced by R_e/B, where B is the proportionality constant relating biosensor response to bound acceptor concentration. The other parameter to emerge from curve fitting of the (R_e, $\overline{C}_A$) data to this rectangular hyperbolic expression is the product $B\overline{\overline{C}}_X$, the maximum response corresponding to saturation of all affinity sites.

The equilibrium constant for the interaction between acceptor and a competing ligand (S) is then obtained by injecting acceptor-ligand mixtures with a range of total concentrations of competitive inhibitor ($\overline{C}_S$). Because the magnitude of $B\overline{\overline{C}}_X$ has already been established, the substitution of each (R_e, $\overline{C}_A$, $\overline{C}_S$) combination into Equation A.5A.27 yields the value of K_{AX} pertinent to Equation A.5A.24. K_{AS} is then evaluated from the slope of the dependence of $Q = K_{AX}/K_{AX}$ upon $[q\overline{C}_S - (Q-1)\overline{C}_A/Q]$.

Analysis of results obtained from the IAsys instrument follows essentially the same protocol, except that $\overline{C}_A$ in Equation A.5A.27 is not the concentration of acceptor placed in the cuvette, because allowance needs to be made for acceptor depletion as the result of AX formation. Thus, the value of $\overline{C}_A$ for use in Equation A.5A.27 (and Equation A.5A.24) must be obtained from the relationship $\overline{C}_A = (\overline{C}_A)_0 - R_e/B$, where $(\overline{C}_A)_0$ is the concentration of acceptor placed in the cuvette at the start of the measurement (Hall and Winzor, 1997, 1998; Edwards et al., 1998).

Illustrative evaluation of K_{AS} by competitive binding assay

The evaluation of the equilibrium constant for an acceptor-ligand interaction by competitive binding assay is illustrated here using a BIAcore study of the interaction between interleukin 6 and the soluble form of its specific receptor (Ward et al., 1995). The recombinant cytokine proved to be dimeric, and has therefore been attached to the biosensor surface so that results could be interpreted in terms of Equation A.5A.27 with the soluble receptor as the univalent acceptor (A). Characterization of the acceptor-matrix interaction is summarized in Figure A.5A.11A, which presents the results in Scatchard format. Values of 2.4 ($\pm$ 0.2) $\times 10^7$ M^{-1} and 2200 ($\pm$ 100) response units are obtained for K_{AX} and the product $B\overline{\overline{C}}_X$, respectively. Results from the series of experiments on mixtures of acceptor and interleukin 6 are plotted in Figure A.5A.11B according to Equation A.5A.24, with $q = 2$ because of the dimeric nature of the cytokine. A value of 4.8 ($\pm$ 0.3) $\times 10^7$ M^{-1} is obtained from the slope.

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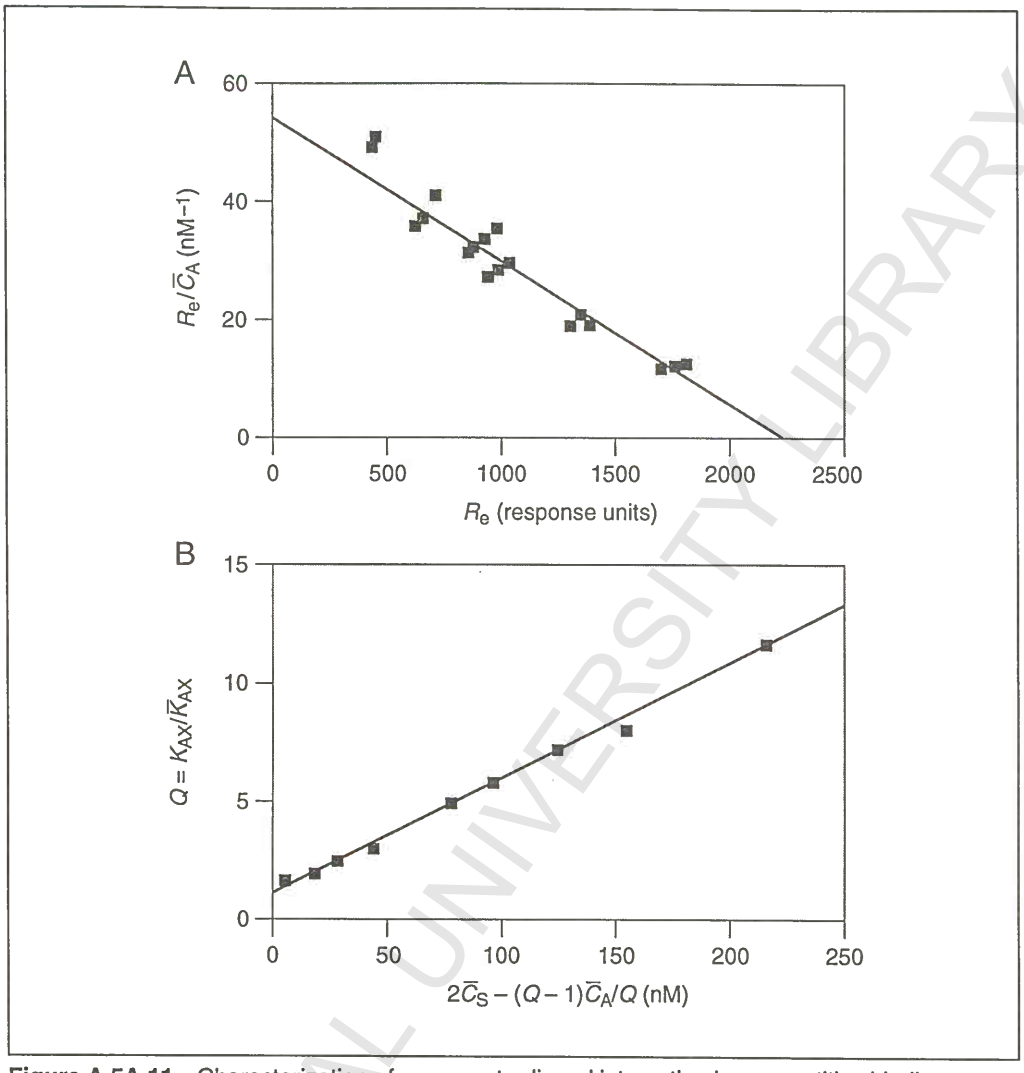

Figure A.5A.11 Characterization of an acceptor-ligand interaction by competitive binding assay. (**A**) Scatchard plot of equilibrium responses for the interaction of the soluble domain of interleukin 6 receptor (A) with interleukin 6 immobilized on the sensor surface of a BIAcore instrument. (**B**) Plot of results obtained with interleukin 6 as the competing ligand (S) in accordance with Equation A.5A.24, with $q = 2$. Data taken from Ward et al. (1995).

This use of BIAcore data to illustrate the evaluation of equilibrium constants by competitive binding assay is open to criticism on the grounds that partition or chromatographic studies are more traditional forms of quantitative affinity chromatography. However, the selected example serves an important role by emphasizing the assumed univalence of acceptor in the analysis, and by illustrating one means by which the problem of multivalence can be averted. An alternative approach is discussed in the next section.

BINDING RESPONSES DEVIATING FROM RECTANGULAR HYPERBOLIC FORM

It has already been emphasized that the rectangular hyperbola represents the simplest form of binding response, and that deviations therefrom signify the need for the incorporation of additional complexity into the model to account for the shape of the binding curves. Possible sources of deviation from the form shown as the broken line (- - -) in Figure A.5A.1 are considered below.

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Effect of Acceptor Site Heterogeneity

Equations A.5A.1-A.5A.4 have been used to describe a model involving ligand interaction with two equivalent and noninteracting acceptor sites; this model can now be extended to a situation in which the two binding sites are nonequivalent but still independent. Independence means that the binding constant for one site is the same regardless of whether the other site is occupied or not. Thus, in terms of Equations A.5A.1-A.5A.4, $K_1 = K_4$ and $K_2 = K_3$. A rearrangement of Equation A.5A.7 shows that the binding to two nonequivalent and independent sites is described by the sum of two rectangular hyperbolae.

$$r = \frac{K_1 C_S (1 + K_2 C_S) + K_2 C_S (1 + K_1 C_S)}{(1 + K_1 C_S)(1 + K_2 C_S)}$$

Equation A.5A.28

$$r = \frac{K_1 C_S}{(1 + K_1 C_S)} + \frac{K_2 C_S}{(1 + K_2 C_S)}$$

Equation A.5A.29

For two nonequivalent groups of sites (p and q in each group) the general binding equation becomes

$$r = \frac{pK_1 C_S}{(1 + K_1 C_S)} + \frac{qK_2 C_S}{(1 + K_2 C_S)}$$

Equation A.5A.30

which may be used directly in nonlinear least-squares fitting programs to extract estimates of the two binding constants and the two site numbers. The corresponding Scatchard plot is nonlinear and of the form shown in Figure A.5A.12. Here, the deviation from linearity is useful in recognizing that a simple model involving homogeneous sites is inadequate to describe the experimental data. For a more quantitative analysis, the Scatchard plot is of limited use. A common misconception is that the limiting slopes of this graph provide estimates of the two binding constants while their extrapolation to the abscissa provides the respective site numbers. This difficulty is illustrated in Figure A.5A.12, which shows the Scatchard plots for a binding model involving two independent sites that differ in affinity by factors of 2, 10, and 100. The Scatchard plots for the binding to each independent site are included. The following features are apparent:

1. The Scatchard plot is not the simple sum of the plots describing the individual site contributions.
2. The abscissa intercept represents the total site number (in this case two), but becomes more difficult to determine graphically as the difference between the binding affinities increases.
3. When the binding affinities differ by a factor of two, it becomes very difficult to experimentally identify the deviation from linearity and thus the site heterogeneity.
4. As the difference between the binding affinities increases, the limiting slope of the steepest part of the Scatchard plot provides an estimate of the tighter binding constant.

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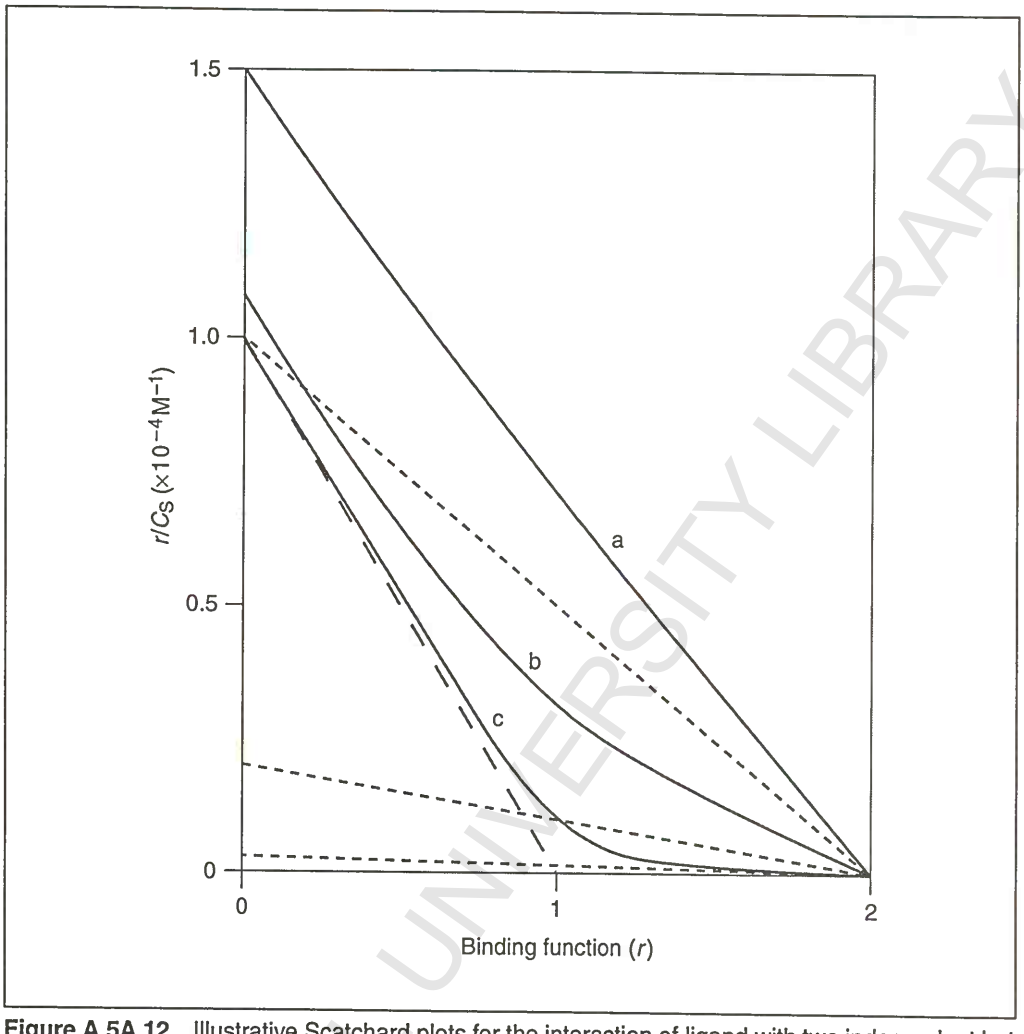

Figure A.5A.12 Illustrative Scatchard plots for the interaction of ligand with two independent but nonequivalent acceptor sites. The higher-affinity site has been ascribed a binding constant of 10^5 M^{-1}, whereas the other has been accorded values of 5×10^4 M^{-1} (curve a), 10^4 M^{-1} (curve b), and 10^3 M^{-1} (curve c). The broken line describes the interaction of ligand with the higher-affinity binding site, whereas the dotted lines have a common abscissa intercept of 2 and slopes characteristic of the weaker interactions.

However, as has been carefully documented by Klotz (1997), the limiting slopes and intercepts at high and low values of r are complex functions of the two binding constants and site numbers.

5. Besides the difficulty of extracting the binding parameters graphically from the Scatchard plot, it should be recognized that a similar deviation from linearity is observed in cases where multiple sites interact in a negatively cooperative manner.

A slightly different situation arises when the two groups of sites (p and q) are on two different noninteracting molecules (A and B) rather than on the same molecule. In addition to being determined by the binding affinities and site numbers (K_{AS}, K_{BS}, p, q), the binding function is determined by the mole fraction (α_i) of each acceptor species present: $\alpha_A = \overline{C}_A/(\overline{C}_A + \overline{C}_B)$ and $\alpha_B = (1 - \alpha_A) = \overline{C}_B/(\overline{C}_A + \overline{C}_B)$, where the overbar notation again signifies the total concentration (free and complexed) of a given acceptor. In these circumstances

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$$r = \frac{\alpha_A p K_{AS} C_S}{(1 + K_{AS} C_S)} + \frac{(1 - \alpha_A) q K_{BS} C_S}{(1 + K_{BS} C_S)}$$

Equation A.5A.31

where

$$\alpha_A = \frac{C_A (1 + K_{AS} C_S)^p}{C_A (1 + K_{AS} C_S)^p + C_B (1 + K_{BS} C_S)^q}$$

Equation A.5A.32

Effect of Ligand Multivalence

A limitation of the binding equations presented thus far has been their reliance upon the assumption that the ligand is univalent in its interaction with acceptor sites. Although the use of those binding equations is entirely reasonable for systems in which the ligand is a small, unifunctional metabolite, the validity of their application to (for example) the interactions of antibodies with their specific surface antigens is highly questionable. The problem is that ligand multivalence introduces a deviation from the rectangular hyperbolic response that is of the same form as that for binding of a univalent ligand to heterogeneous acceptor sites (Calvert et al., 1979). Effects of ligand multivalence on the interpretation of binding data were first considered in the context of quantitative affininty chromatography (Nichol et al., 1981), but have been incorporated subsequently into a general counterpart of the Scatchard analysis (Hogg and Winzor, 1985).

For a situation in which a ligand possessing f sites binds to an acceptor with p sites for ligand, the binding function needs to be defined (Hogg and Winzor, 1985) as

$$r_f = \left(\overline{C}_S^{1/f} - C_S^{1/f}\right) / \overline{C}_A$$

Equation A.5A.33

In terms of this binding function, the multivalent counterpart of the Scatchard equation for equivalent and independent binding governed by the intrinsic association constant K_{AS} is

$$r_f / C_S^{1/f} = pK_{AS} - fK_{AS} r_f \overline{C}_S^{(f-1)/f}$$

Equation A.5A.34

A linear dependence of $r_f / C_S^{1/f}$ upon $r_f \overline{C}_S^{(f-1)/f}$ is thus the requirement for equivalence and independence of sites when the ligand is multivalent.

Because the application of Equation A.5A.34 to binding data requires the specification of a value for f, there has been a tendency to avoid the necessity of so doing by retaining the conventional Scatchard analysis (Equation A.5A.12). However, such action merely signifies that a value of unity has been chosen as the most appropriate valence of the ligand. Results from a partition equilibrium study of the binding of aldolase to muscle myofibrils (Kuter et al., 1983) serve to emphasize that point. The conventional Scatchard analysis of those results is presented in Figure A.5A.13A, which seemingly signifies heterogeneity of the myofibrillar sites in their interaction with enzyme. However, symptoms of site heterogeneity disappear on reanalysis of the results according to Equation

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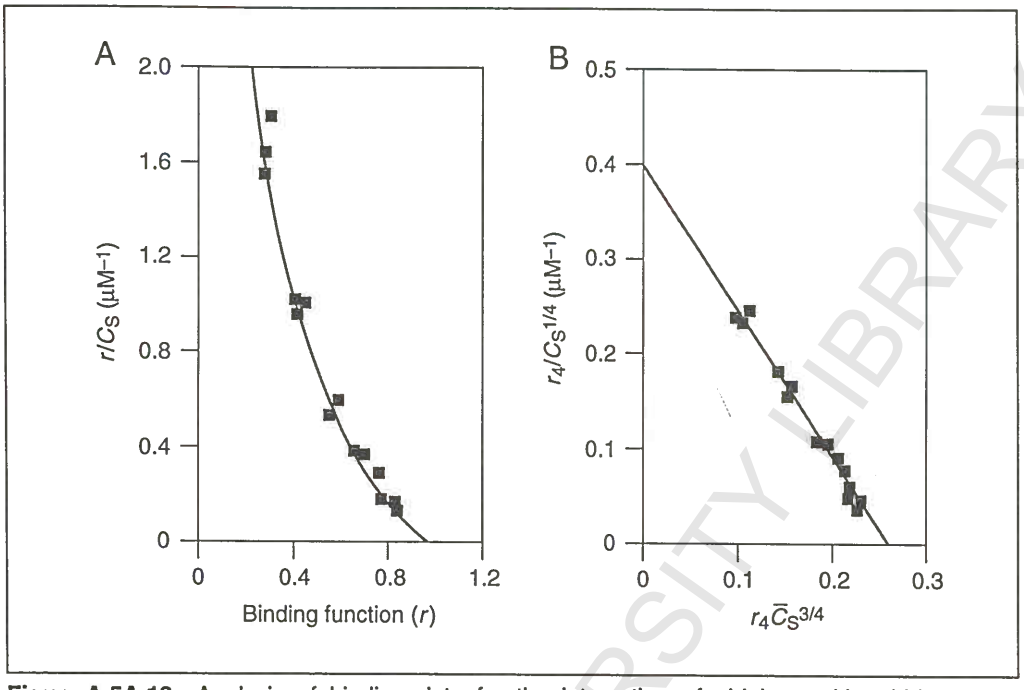

Figure A.5A.13 Analysis of binding data for the interaction of aldolase with rabbit muscle myofibrils. (**A**) Conventional Scatchard plot. (**B**) Multivalent Scatchard plot according to Equation A.5A.34 with $f = 4$. Data taken from Table 1 of Kuter et al. (1983).

A.5A.34 with $f = 4$ on the grounds that aldolase is a tetramer (Fig. A.5A.13B). The linearity of this plot, particularly in the light of its compliance with the mandatory abscissa intercept of 0.25 (p/f with $p = 1$), establishes the adequacy of a single intrinsic binding constant of 3.8 (± 0.3) × 10^5 M^{-1} to describe the binding of tetravalent enzyme to sites on the myofibrillar matrix.

Although this analysis is open to criticism on the grounds of its reliance upon transformed experimental parameters, the finding has been confirmed by analysis of the results in terms of the rectangular hyperbolic expression of which Equation A.5A.34 is the linear transform (Harris et al., 1995a). Use of the expression

$$fr_f\bar{C}_S^{(f-1)/f} = \frac{pf\bar{C}_S^{(f-1)/f}K_{AS}C_S^{1/f}}{1+f\bar{C}_S^{(f-1)/f}K_{AS}C_S^{1/f}}$$

Equation A.5A.35

allows the magnitudes of p and K_{AS} to be obtained by substituting $fr_f\bar{C}_S^{(f-1)/f}$ for r and $f\bar{C}_S^{(f-1)/f}C_S^{1/f}$ for C_S in standard computer programs for the evaluation of binding parameters.

In studies of binding to a particulate acceptor there are circumstances under which a rectangular hyperbolic binding response is observed despite multivalence of the ligand (Nichol et al., 1974; Brinkworth et al., 1975)—a finding rationalized intuitively at the time on the grounds that physical restrictions precluded multiple interactions of ligand with acceptor. That the binding constant deduced under such circumstances is the product fK_{AS} has been confirmed by a subsequent mathematical explanation of such behavior (Kalinin et al., 1995), which arises when the total concentration of ligand greatly exceeds that of acceptor sites. Under conditions where the concentration of bound ligand ($\bar{C}_S$ –

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C_S) is small relative to the free concentration (C_S), the total ligand concentration $\overline{C}_S$ may be written as:

$$\overline{C}_S = C_S + (\overline{C}_S - C_S) = C_S(1+\delta)$$

Equation A.5A.36

where $\delta = (\overline{C}_S - C_S)/C_S$ is much smaller than unity. From the binomial theorem it follows that

$$\overline{C}_S^{1/f} = C_S^{1/f}(1+\delta/f+\ldots)$$

Equation A.5A.37

whereupon the binding function (Equation A.5A.33) becomes $r_f = C_S{}^{1/f}\delta/(f\overline{C}_A)$, and Equation A.5A.34 simplifies to

$$(\overline{C}_S - C_S)/C_S = fK_{AS}\overline{C}_A - fK_{AS}(\overline{C}_S - C_S)[1+(f-1)\delta/f]$$

Equation A.5A.38

which signifies a slope of $f[1 + (f - 1)\delta/f]K_{AS} \approx fK_{AS}$ for the slope of the conventional Scatchard plot.

Equivalent but Cooperative Binding

In instances where the acceptor comprises several identical (or very similar) subunits, the binding of a univalent ligand to any one subunit has the potential to induce conformational changes in adjacent subunits and thereby alter their intrinsic affinities for ligand. This concept of equivalent but dependent acceptor sites was introduced by Koshland et al. (1966), who noted the existence of two possible situations. (1) In positive cooperativity, ligand-induced conformational changes in the acceptor enhance subsequent ligand attachments, resulting in a sigmoidal binding response (Fig. A.5A.14A). (2) In negative cooperativity, the alternative situation occurs, with a decreased intrinsic binding constant as the result of conformational change(s) giving rise to a binding response of the same form as that reflecting heterogeneity of acceptor sites (Fig. A.5A.14B).

Allowance for nonidentity of successive intrinsic association constants is effected in the Adair (1925) formulation of the binding expression. Thus, Equation A.5A.7 for a system with $p = 2$ becomes:

$$r = \frac{2(K_{AS})_1 C_S + 2(K_{AS})_1(K_{AS})_2 C_S^2}{1+2(K_{AS})_1 C_S + (K_{AS})_1(K_{AS})_2 C_S^2}$$

Equation A.5A.39

where $(K_{AS})_1$ is the intrinsic binding constant for the interaction of S with either site on free A, and $(K_{AS})_2$ is the modified value for occupancy of the remaining site to complete saturation of the acceptor.

Multivalence of the ligand introduces the need to consider whether acceptor or ligand sites are the source of the cooperativity (Harris et al., 1995b). For systems in which the cooperativity resides in acceptor sites, the criteria used above for the interpretation of

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binding curves for univalent ligands (Fig. A.5A.14) also apply to data for multivalent ligands provided that results are analyzed in terms of the rectangular hyperbolic relationship for an *f*-valent ligand (Equation A.5A.35) or its linear transform (Equation A.5A.34). Thus, bivalent Scatchard plots of the form shown in Figure A.5A.15, panels A and B, signify the existence of positive and negative cooperativity, respectively. However, the interpretation must be reversed when ligand sites are the source of cooperativity: the sigmoidal response (Fig. A.5A.15A) now signifies negatively cooperative binding (Harris et al., 1995b).

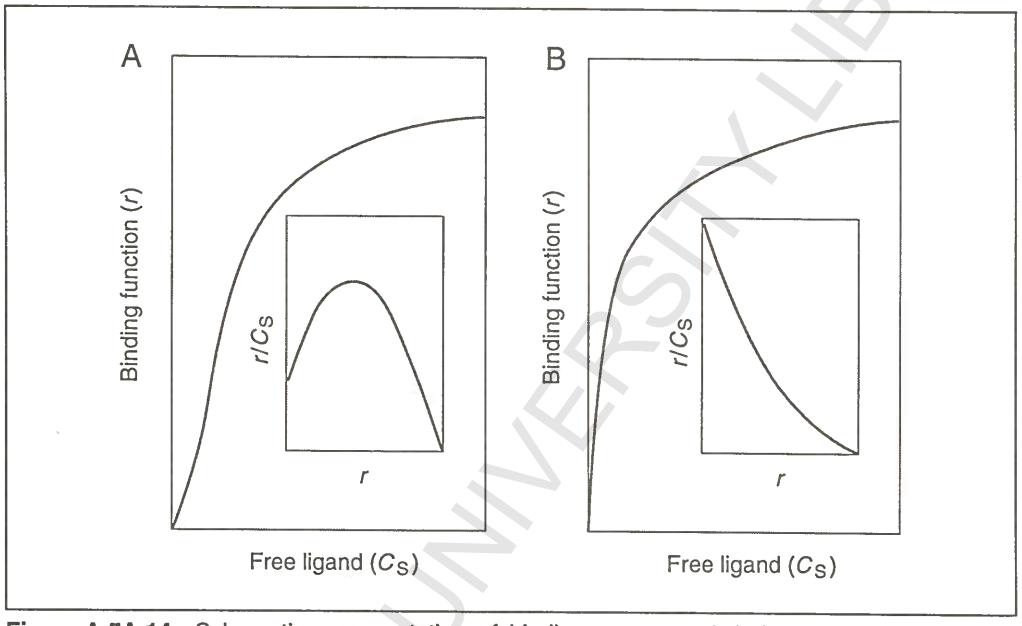

Figure A.5A.14 Schematic representation of binding curves and their Scatchard transforms reflecting **(A)** positive cooperativity and **(B)** negative cooperativity of acceptor sites for a univalent ligand.

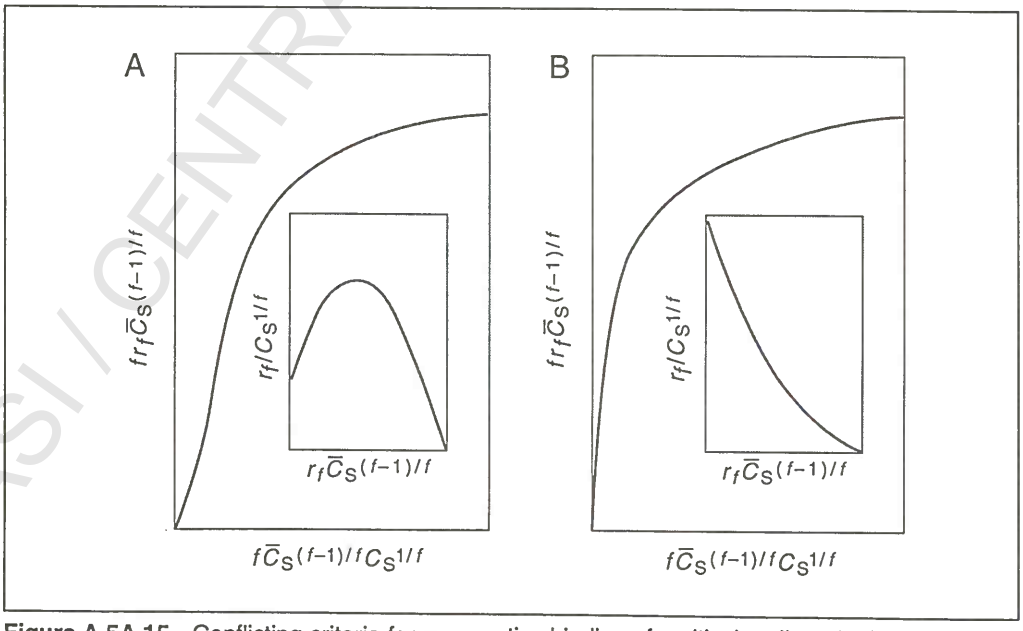

Figure A.5A.15 Conflicting criteria for cooperative binding of multivalent ligands. **(A)** Schematic binding curve and its multivalent Scatchard counterpart reflecting either positive cooperativity of acceptor sites or negative cooperativity of ligand sites. **(B)** Corresponding plots reflecting either negative cooperativity of acceptor sites or positive cooperativity of ligand sites.

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Because conflicting criteria apply to the interpretation of cooperativity emanating from acceptor and ligand sites, additional information is clearly required before mechanistic significance can be ascribed to the forms of binding curves or their linear transforms. In that regard, the interpretation of results obtained for the binding of pyruvate kinase to muscle myofibrils (Harris et al., 1995b) serves to illustrate the type of approach that may be used to resolve the dilemma.

Interplays of Equilibria

Instead of considering the acceptor to undergo ligand-induced conformational changes, Monod et al. (1965) propose that a sigmoidal binding response reflects the preferential binding of a univalent ligand to one of two isomeric acceptor states that coexist in equilibrium. Although the binding sites within a given acceptor state are considered to be equivalent and independent, the intrinsic binding constant and/or the number of binding sites are allowed to differ between the two isomeric states. Even greater generality is accorded this model by allowing the preexisting equilibrium to include acceptor self-association (Nichol et al., 1967). For a system where states A and C possess p and q sites, respectively, with respective intrinsic association constants K_{AS} and K_{CS}, the binding equation becomes:

$$r = \frac{pK_{AS}C_S(1+K_{AS}C_S)^{p-1} + nXC_A^{n-1}qK_{CS}C_S(1+K_{CS}C_S)^{q-1}}{(1+K_{AS}C_S)^{p} + nXC_A^{n-1}(1+K_{CS}C_S)^{q}}$$

Equation A.5A.40

where C_A and C_C are the concentrations of the respective isomeric states, and $X = C_C/C_A{}^n$ is the equilibrium constant describing the preexisting coexistence of acceptor states (nA in equilibrium with C).

Preferential binding to an isomerizing acceptor

A major difference between the models based on acceptor isomerization and self-association is the dependence of the binding function upon acceptor concentration for $n > 1$, but its independence of acceptor concentration for the Monod et al. (1965) model ($n = 1$ in Equation A.5A.40). The latter feature is shared by the cooperativity model (Koshland et al., 1966), which is mathematically equivalent to the Monod model of allostery in the absence of a means of distinguishing between an initial state ($C_S = 0$) comprising a single acceptor state (Koshland et al., 1966) and an initial state comprising an equilibrium mixture of isomeric states with one form dominant (Monod et al., 1965).

Under favorable circumstances the molecular crowding effect of thermodynamic nonideality has potential for making such distinction (Harris and Winzor, 1988; Bergman and Winzor, 1989; Bergman et al., 1989), but pyruvate kinase is the only allosteric system to which this method has been applied, and thereby established preexistence of an isomerization equilibrium (Harris and Winzor, 1988). However, even that approach is not necessarily definitive, because failure to observe a nonideality-mediated shift towards a smaller isomeric state does not preclude the possibility that the conformation change relating to a preexisting isomerization equilibrium is too subtle for detection as a gross change in size and/or shape. Furthermore, as noted by Saroff (1991), the two models are not mutually exclusive inasmuch as coexisting acceptor states may also exhibit cooperativity of ligand binding.

In view of the above considerations, a logical approach is to set aside aspirations of an unequivocal and mechanistically correct characterization of a sigmoidal binding response

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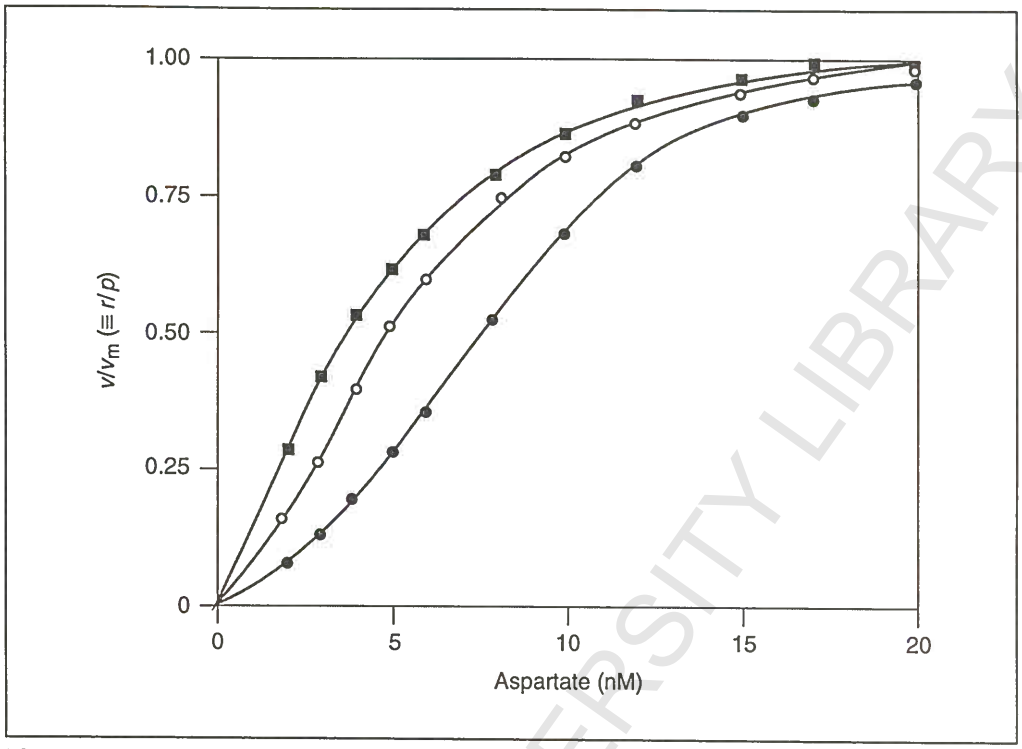

Figure A.5A.16 Allosteric activation and inhibition of *E. coli* aspartate transcarbamylase by effectors. Open circles, dependence of initial velocity v (expressed relative to maximal value v_m) upon aspartate concentration (C_S) in the presence of saturating carbamyl phosphate; filled circles and filled squares, corresponding dependences with 0.5 mM CTP and 2 mM ATP, respectively, included in the reaction mixtures. Data taken from Gerhart (1970).

in favor of an acceptable thermodynamic description in terms of the allosteric model for which parameters are evaluated more readily. The authors' inclination is towards interpretation in terms of the Monod model, which is also adapted readily to incorporate the consequences of allosteric effectors.

The moderating influence of an effector (E) binding to different sites from S on the two isomeric states is accommodated in Equation A.5A.40 with $n = 1$ by replacing the self-association constant (X) with a constitutive isomerization constant ($\overline{X}$) defined as:

$$\overline{X} = \frac{X(1+K_{CS}C_E)^z}{(1+K_{AE}C_E)^w}$$

Equation A.5A.41

where w and z are the respective numbers of sites for effector on A and C isomers, for which intrinsic binding constants of K_{AE} and K_{CE} pertain (Frieden, 1967; Changeux and Rubin, 1968; Nichol et al., 1972b). Preferential binding of E to the isomer with a lower affinity for S gives rise to allosteric inhibition and a more sigmoidal binding curve in the presence of effector. Conversely, the binding curves exhibit activation and a tendency to become rectangular hyperbolic when the effector binds preferentially to the isomer with higher affinity for S. These phenomena are evident in Figure A.5A.16, which illustrates the inhibitory effect of CTP and the activatory effect of ATP on the aspartate transcarbamylase reaction (Gerhart, 1970).

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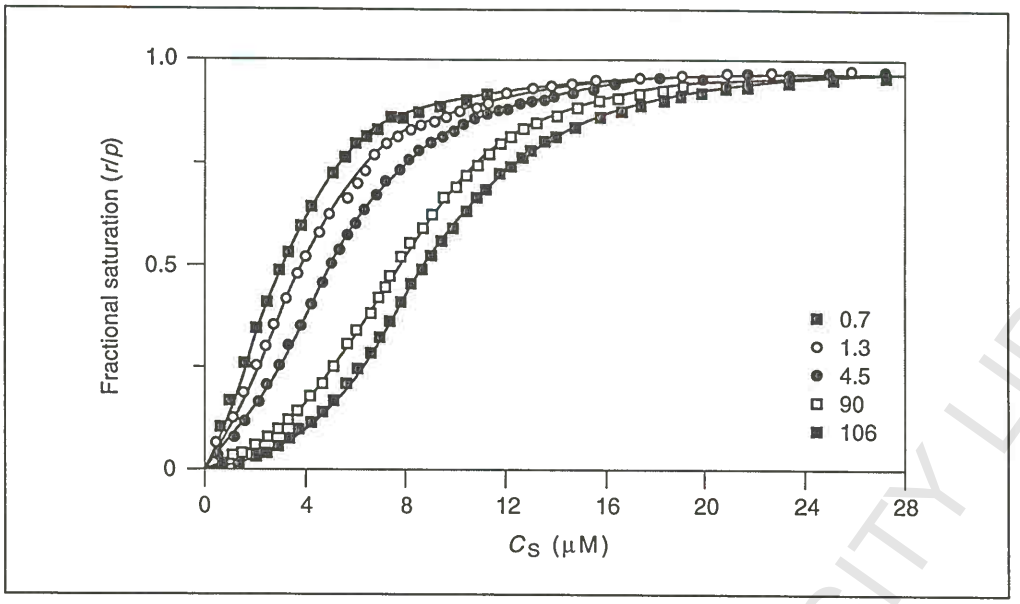

Figure A.5A.17 Concentration dependence of the binding curve for a self-associating acceptor. Binding curves for oxygen uptake by the indicated total concentrations of human hemoglobin (0.7 to 106 μg/ml) at pH 7.4. Data taken from Mills et al. (1976).

Preferential binding to a self-associating acceptor

Whereas the binding function for the interaction of a ligand with an isomerizing acceptor is expressed in terms of free ligand concentration (C_S) as the single variable, its magnitude also depends upon the free acceptor concentration (C_A), and hence the total acceptor concentration when $n > 1$ in Equation A.5A.40. In that regard, the required concentration of free monomer is given by the physically acceptable solution of the equation expressing mass conservation of acceptor, namely:

$$nX(1+K_{CS}C_S)^q C_A^n + (1+K_{AS}C_S)^p C_A - \overline{C}_A = 0$$

Equation A.5A.42

where $\overline{C}_A$ is the base-molar concentration of acceptor (total weight concentration divided by the molecular weight of monomer). Simulations of a binding curve also require the specification of magnitudes for the stoichiometry (n) and equilibrium constant (X) describing the preexisting self-association of acceptor—parameters obtained by physicochemical characterization of the acceptor in the absence of ligand (Nichol et al., 1972a; Tellam et al., 1978, 1979).

The importance of maintaining a fixed total acceptor concentration during the experimental delineation of a binding curve for a self-associating acceptor is evident from Figure A.5A.17, which summarizes the interaction of oxygen with hemoglobin over a wide range of protein concentrations (0.7 to 106 μg/ml)—a range where the tetrameric $\alpha_2\beta_2$ species coexists in equilibrium with dimer ($\alpha\beta$), which binds oxygen preferentially (Mills et al., 1976). Clearly, there is a separate binding curve for each fixed total acceptor concentration. Although a change in $\overline{C}_A$ may well enhance the accuracy with which a value of r may be determined at a given C_S, a binding curve constructed in this manner would comprise a composite of segments from the binding curves described by Equation A.5A.40 for each total concentration of acceptor used.

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Sigmoidal effects reflecting ligand self-association

There is a tendency to regard the acceptor as the species undergoing self-association or isomerization, and therefore to regard allostery as a property emanating from the acceptor. However, there are also situations in which the reactant undergoing self-interaction is the ligand. For the relatively simple situation in which each state of a self-associating ligand ($n\mathrm{S} \leftrightarrow \mathrm{T}$) interacts competitively with the same p equivalent and independent sites on an acceptor A for which the respective intrinsic binding constants are K_{AS} and K_{AT}, it is relevant to define the binding function as:

$$r = \left(\overline{C}_S - C_S - nC_T\right)/\overline{C}_A$$

Equation A.5A.43

whereupon the binding equation becomes

$$r = \frac{p\left(K_{AS}C_S + nK_{AT}C_T\right)}{1 + K_{AS}C_S + K_{AT}C_T}$$

Equation A.5A.44

A rectangular hyperbolic response of r upon $\overline{C}_S = C_S + nC_T$ results from ligand isomerization, whereas a sigmoidal response emanates when $n > 1$ and $K_{AS} = 0$, the situation when acceptor binds exclusively to the polymeric form of the ligand. The curves exhibit symptoms of negative cooperativity when monomeric ligand is the exclusive form of ligand bound (Nichol et al., 1969). An example of a situation in which the polymeric form of ligand binds preferentially to acceptor is provided by the interaction of chlorpromazine with tubulin (Hinman and Cann, 1976; Cann et al., 1981)—a system for which the Scatchard plot exhibits two critical points, a maximum and a minimum. Although the unusual form of the Scatchard plot was initially interpreted as signifying relatively strong interaction of chlorpromazine with a single tubulin site and weaker, positively cooperative binding of an additional eight or nine chlorpromazine molecules (Hinman and Cann, 1976), it has subsequently been shown that preferential binding of the chlorpromazine micelle ($n = 9$ to 11) is a more plausible explanation of the results (Cann et al., 1981).

Effect of Ligand Binding on Acceptor Self-Association

For an acceptor undergoing preexisting self-association between monomer and a single higher polymeric state (nA in equilibrium with C), the association equilibrium constant can be determined from studies of the acceptor in the absence of ligand. For example, the weight-average molecular weight ($\overline{M}_W$) of an equilibrium mixture with total acceptor concentration ($\overline{c}_a$ in mg/ml) is given by:

$$\overline{M}_W = \left[c_A M_A + \left(\overline{c}_a - c_A\right) n M_A\right]/\overline{c}_a$$

Equation A.5A.45

where M_A is the molecular weight of the monomer. This allows the concentration of monomeric acceptor (c_A) to be evaluated from the expression

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$$c_A = \frac{\bar{c}_a(nM_A - M_w)}{(n-1)M_A}$$

Equation A.5A.46

for an assigned value of n. In fact, this specification of the association stoichiometry can be avoided by direct analysis of the sedimentation equilibrium distributions reflecting acceptor self-association (Milthorpe et al., 1975; Wills et al., 1996).

The availability of methods for determining c_A as a function of total acceptor concentration allows the association equilibrium constant X (liter^{n-1}mol^{n-1}) to be determined as:

$$X = \left[(\bar{c}_a - c_A)/c_A^n\right]\left[M_A^{n-1}/n\right]$$

Equation A.5A.47

This is frequently accomplished from a logarithmic variant of that relationship, namely

$$\log(\bar{c}_a - c_A) = \log\left(nX/M_A^{n-1}\right) + n\log c_A$$

Equation A.5A.48

which allows the magnitudes of n and X to be determined from the slope and ordinate intercept of the linear dependence of $\log(\bar{c}_a - c_A)$ upon $\log c_A$.

Provided that the ligand is sufficiently small in comparison with acceptor for the approximations $M_{AS_i} \approx M_C$ and $M_{CS_i} \approx M_C$ to apply, the perturbation by ligand of the self-association equilibrium affords quantitative information on the relative affinities of the two acceptor states for S (Nichol et al., 1972a; Baghurst and Nichol, 1975; Tellam et al., 1979). Under those circumstances the determination of molecular weight data or direct analysis of sedimentation equilibrium distributions yields $\bar{c}_A$, the weight-concentration of monomer in free and ligand-bound states, whereupon the parameter obtained from Equation A.5A.44 is a constitutive association equilibrium constant ($\bar{X}$). Its relationship to parameters within the interplay of equilibria is as follows:

$$\bar{X} = (\bar{c}_a - c_A)/c_A^n = \frac{X(1 + K_{CS}C_S)^q}{(1 + K_{AS}C_S)^{np}}$$

Equation A.5A.49

The use of this approach is illustrated by a study of the interaction between indole and α-chymotrypsin (Ford and Winzor, 1983), which undergoes reversible dimerization under the conditions of the investigation—a factor evident from the slope of 2 for the plot of sedimentation equilibrium results in the absence of ligand (Fig. A.5A.18, open symbols) according to Equation A.5A.48. Repetition of the experiments with enzyme in dialysis equilibrium with 1 mM (filled circles) and 2 mM (filled squares) indole gives rise to a progressive decrease in $\bar{X}$ that is commensurate with monomer exhibiting a five-fold greater affinity than dimeric α-chymotrypsin for ligand ($p = 1$, $q = 2$, $K_{AS} = 5K_{CS}$).

Interplays of equilibria involving acceptor self-association can be characterized with far greater certainty and precision than those involving acceptor states in isomerization equilibrium. Studies of the acceptor in the absence of ligand can usually be used to guide the distinction between preexisting and ligand-induced self-association as the source of

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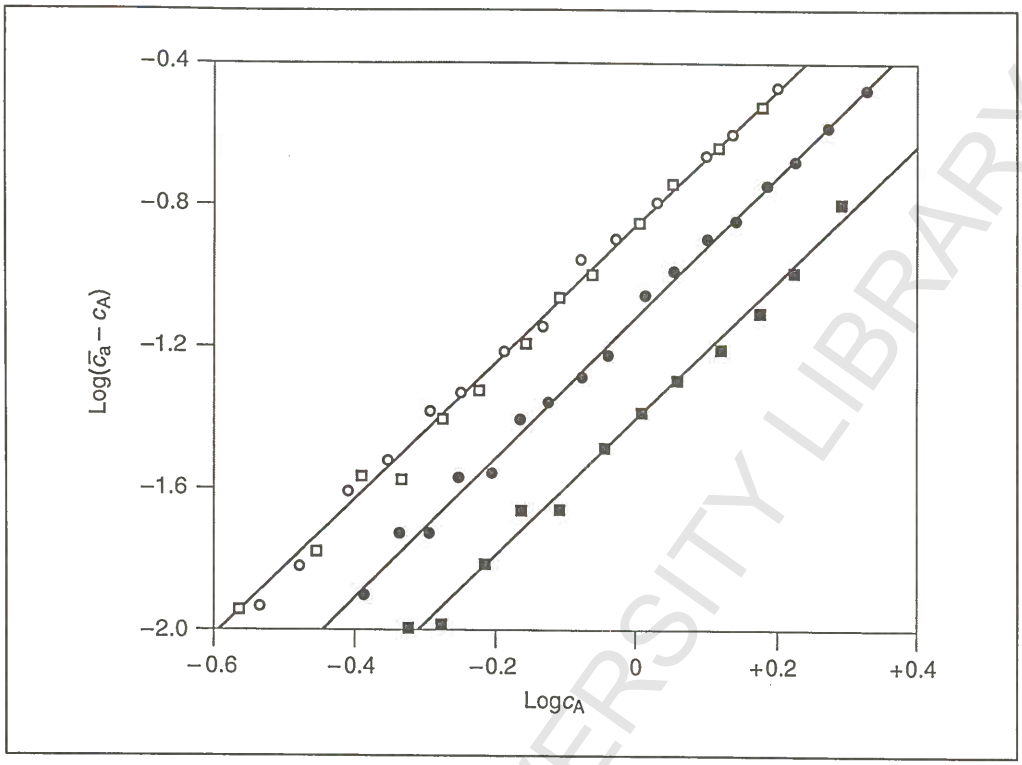

Figure A.5A.18 Perturbation of the monomer-dimer equilibrium for α-chymotrypsin (pH 3.9, *I* 0.11) by the preferential interaction of indole with monomeric enzyme. Plot of results from sedimentation equilibrium experiments according to Equation A.5A.48 for enzyme alone (open symbols) and in the presence of 1 mM (filled circles) and 2 mM (filled squares) ligand.

a sigmoidal binding curve (e.g., in Fig. A.5A.18, where the dimerization of α-chymotrypsin is clearly a preexisting equilibrium). Furthermore, perturbations of the preexisting self-association equilibrium as the result of preferential binding to one acceptor state provides additional quantitative information on the relative affinities of the two acceptor states for ligand. Indeed, measurement of the effect of free ligand concentration on the magnitude of $\overline{X}$ has formed the sole basis for characterization of the interaction between NADH (an analog of 2,3-bisphosphoglycerate) and methemoglobin—a system for which no meaningful parameters would be extracted from analysis of the binding curve (Jacobsen and Winzor, 1995). On the other hand, the characterization of an interplay of equilibria involving acceptor isomerization relies almost entirely on the analysis of the binding curve—a situation that leads to the isomerization constant, as well as the binding characteristics for the isomer with lower affinity for ligand, being curve-fitting parameters. To that end, the use of thermodynamic nonideality effected by the inclusion of molecular crowding agents to probe preexisting isomerization equilibria can, under favorable circumstances, not only detect the phenomenon but also yield an independent estimate of *X* (Harris and Winzor, 1988).

COMPLICATIONS ARISING FROM NONSPECIFIC BINDING

There are many examples in the literature where binding curves deviate from rectangular hyperbolic form as the result of elements of nonspecificity in the overall interaction between ligand and acceptor. There are two major areas where nonspecificity is encountered, and in each the term nonspecific binding has a different connotation.

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Membrane-Receptor Interactions

The binding of ligands to membrane-bound acceptors (receptors) is a fundamental phenomenon encountered in cell biology, immunology, and endocrinology. Many studies of these ligand-receptor interactions entail the use of radioactively labeled ligand to facilitate measurement of the concentration of unbound ligand in the supernatant after centrifugation to pellet the membranes. Results of such experiments rarely exhibit a rectangular hyperbolic dependence of binding upon free ligand concentration inasmuch as no plateau region is reached. Instead, there is a continuous increase in binding, which is seemingly unsaturable (filled squares, Fig. A.5A.19A). An operative criterion that is used to distinguish specific interaction from other phenomenon, termed nonspecific binding, relies upon an assertion that specifically bound ligand can be displaced by an excess of nonradioactively labeled ligand, whereas the other form of binding is unaffected by this procedure (filled circles, Fig. A.5A.19A).

The above definition of nonspecific binding only covers ligand-membrane interactions that are irreversible. In that regard, binding curves of the same form as that observed for

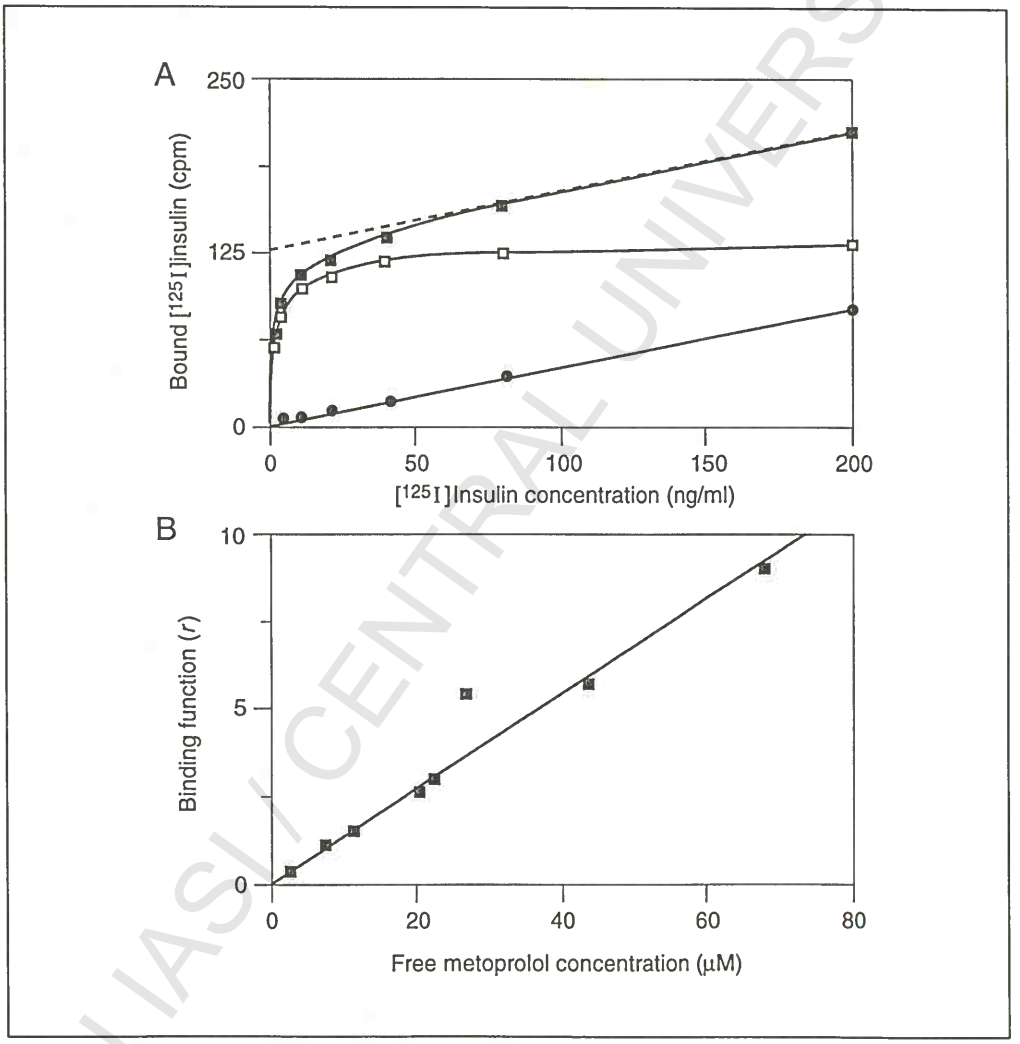

Figure A.5A.19 Nonspecific ligand interactions in studies involving membrane receptors. (**A**) Binding of [^{125}I]insulin to insulin receptors on Chinese hampster ovary cells (filled squares), and the corrected binding curve (open squares) after allowance for bound radioactivity in the presence of excess nonlabeled insulin (filled circles). Adapted from Winzor and Sawyer (1995). (**B**) Uptake of metoprolol as the result of physical partition into hepatic microsomes. Data taken from Bogoyevitch et al. (1987).

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the interaction of insulin with its membrane receptors can also reflect nonspecific uptake of ligand as the result of partition into the membrane phase. Such ligand partitioning has a similar effect on the form of the binding curve because its dependence upon free ligand concentration is also linear (Fig. A.5A.19B). However, inasmuch as the partitioned ligand can be displaced by an excess of nonradioactively labeled ligand, the phenomenon would be classified as specific binding by the above definition.

A convenient procedure for the analysis of systems exhibiting both specific binding and partition requires the collection of binding data at concentrations well in excess of those required for saturation of acceptor sites—a region where the expression for binding assumes the form

$$r\overline{C}_A = \overline{C}_S - C_S = K_p C_S + p\overline{C}_A$$

Equation A.5A.50

where multiplication of the binding function by $\overline{C}_A$ overcomes the problem that the total concentration of receptor sites is one of the parameters to be evaluated from the analysis. From Equation A.5A.50 it is evident that extrapolation of the linear part of the curve at high values of C_S to the ordinate provides the value of $p\overline{C}_A$, the concentration of bound ligand associated with saturation of receptor sites, whereas the partition coefficient (K_p) is obtained from the slope. Once the partition contribution has been defined quantitatively, it can be subtracted from the overall binding curve to allow analysis of the corrected binding curve by standard procedures. Such a procedure can thus be applied to binding curves of the form shown in Figure A.5A.19A, irrespective of the phenomenon responsible for the nonsaturable binding (Winzor and Sawyer, 1995).

Protein-Polymer Interactions

Interactions between proteins and linear polymer chains such as polynucleotides (Latt and Sober, 1967; McGhee and von Hippel, 1974) and polysaccharides (Olson et al., 1991; Munro et al., 1998) give rise to another form of nonspecificity whenever the site for ligand binding comprises a sequence of two or more consecutive residues on the polymer chain. Because of the similarity of the repeating units, there are no specific acceptor sites, as any residue sequence of the appropriate length is a potential candidate for ligand attachment. Binding curves for such systems are symptomatic of equivalent but negatively cooperative acceptor sites (Fig. A.5A.20A). However, whereas negative cooperativity is usually taken to signify an occupancy-dependent decrease in the affinity of acceptor sites, the phenomenon in protein-polymer interactions merely reflects an occupancy-dependent decrease in the concentration of free acceptor sites that exceeds the concentration of bound ligand.

For the binding of ligand to a sequence of m residues of an N-residue acceptor lattice that is governed by intrinsic association constant K_{AS}, the general form of the binding equation (Epstein, 1978) is:

$$r = \frac{\sum_{i=1}^{i=p} i^h C_i (K_{AS} C_S)^i}{1 + \sum_{i=1}^{i=p} i^h C_i (K_{AS} C_S)^i}$$

Equation A.5A.51

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where p is the integer value of N/m, and ${}^{h}C_{i}$ is the number of possible arrangements of the h items (bound ligands and unoccupied lattice residues) available for distribution on the lattice (Latt and Sober, 1967). The latter is given by:

$$ {}^{h}C_{i} = (N - mi + 1)!/[i!(N - mi)!] $$

Equation A.5A.52

This combinatorial approach for derivation of the binding equation becomes progressively more cumbersome with increasing length of the acceptor lattice. Furthermore, the need to specify N precludes its use, for example, with DNA as the linear polymer chain because

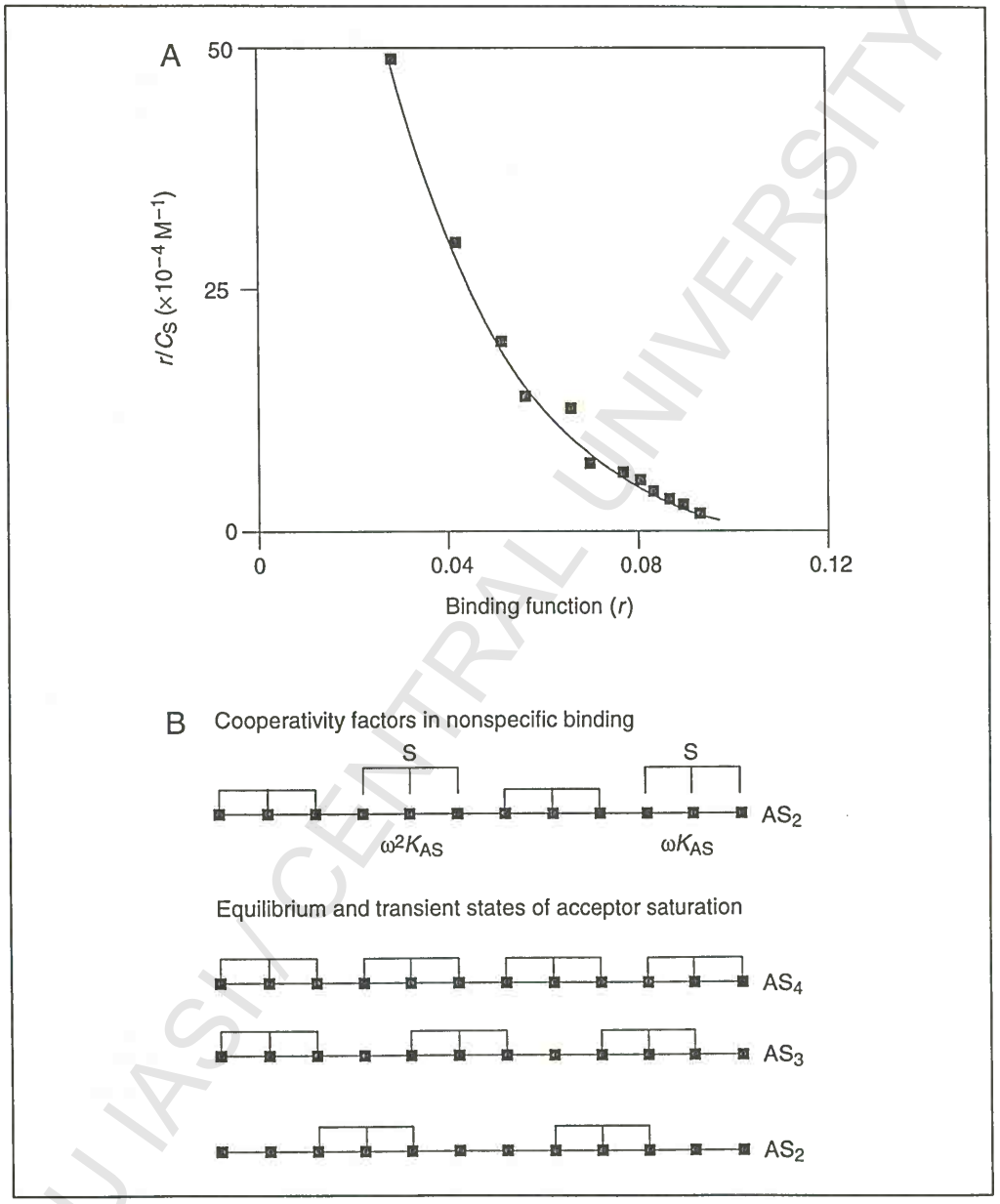

Figure A.5A.20 Effects of nonspecificity in the binding of ligands to a sequence of residues on a linear polymer chain. (**A**) Scatchard plot of experimental results (Latt and Sober, 1967) for the interaction of an ε-dinitrophenyl-labeled hexameric lysine oligopeptide with poly(I + C). (**B**) Schematic representation of thermodynamic and kinetic aspects of nonspecific interaction between a ligand and three-residue sequences on a twelve-residue linear polymer.

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the number of nucleotides comprising the chain is frequently unknown and almost certainly variable as the result of heterogeneity with respect to polynucleotide chain length. These difficulties may be overcome by switching to a probability approach, which only requires N to be a fairly large number. For the random binding of ligand to m-residue sequences of an infinite lattice, the Scatchard transform of the binding equation (McGhee and von Hippel, 1974) is:

$$r/C_S = K_{AS}(1-mr)\left(\frac{1-mr}{1-(m-1)r}\right)^{m-1}$$

Equation A.5A.53

which also has the advantage of being a closed solution.

Conventional cooperativity is incorporated into the model by designating the modified affinity of a sequence adjacent to a bound ligand as ωK_{AS}, the product of the usual intrinsic binding constant and a dimensionless factor ω, and designating that for a flanked sequence as $\omega^2 K_{AS}$ (Fig. A.5A.20B). The Scatchard formulation of the probability treatment of nonspecific binding (McGhee and von Hippel, 1974) then becomes:

$$r/C_S = \frac{K_{AS}(1-mr)\left[(2\omega+1)(1-mr)+r-R\right]^{m-1}\left[1-(m+1)r+R\right]^2}{\left[2(\omega-1)(1-mr)\right]^{m-1}\left[2(1-mr)\right]^2}$$

Equation A.5A.54

where the term R is presented separately for simplification of the typestting of Equation A.5.54.

$$R = \left\{\left[1-(m+1)r\right]^2 + 4\omega r(1-mr)\right\}^{1/2}$$

Equation A.5A.55

Values of ω greater and less than unity signify classical positive and negative cooperativity, respectively. The application of Equation A.5A.54 is addressed in depth by Kowalczykowski et al. (1986).

Finally, it should be noted that there is a kinetic consideration that may affect the attainment of equilibrium in the binding of ligand to a sequence of acceptor residues—a factor evident from the lower part of Figure A.5A.20B for the binding of ligand to three-residue sequences on a twelve-unit lattice. Clearly, the maximum number of ligands that can attach to the chain is four, but random binding can lead to situations in which the lattice is effectively saturated with only three and even two ligand molecules attached (Fig. A.5A.20B). From the thermodynamic viewpoint, these latter situations are not viable options because they merely represent transient states en route to the equilibrium state of four ligand molecules bound at chain saturation. However, although the time-course of equilibrium attainment is irrelevant to the thermodynamics of nonspecific ligand binding, the experimenter must ensure that sufficient time be allowed for the kinetics of bound ligand rearrangement to take its course before the equilibrium measurements are made for substitution into Equations A.5A.51-A.5A.55 (Epstein, 1979; Munro et al., 1998). In that regard, the kinetic problem is maximal for high levels of acceptor saturation, and therefore impinges on the evaluation of m, the sequence length to which ligand binds. Indeed, because this parameter is often deduced under conditions where the acceptor-li-

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gand interaction is stoichiometric, the endpoint of the titration reflects irreversible rather than equilibrium binding. In such titrations there is effectively no opportunity for the dissociation that is necessary to relocate bound ligate molecules and hence achieve maximal efficiency of site occupancy—the requirement for the equilibrium situation. An algorithm is now available for determining the equilibrium endpoint from the measured, irreversible endpoint of a stoichiometric titration (Munro et al., 1998).

CONCLUDING REMARKS

In seeking to explain the behavior of plants and animals at a molecular level, biochemists usually adopt a simple reductionist approach and proceed towards an understanding of in vivo complexity. This transition involves a definition of the in vivo state and eventually the matching of in vitro conditions with those existing in biological tissues or fluids. Since specific recognition mechanisms and binding affinities control the biochemical dynamics of living systems, the thermodynamic binding parameters determined under in vitro conditions must be matched to those pertaining to living systems. The acquisition and quantitative analysis of binding data is therefore an essential part of this process.

Nature has evolved a variety of complex mechanisms to deal with the interleaving and interdependence of biochemical reactions. While the simple binding function (Equation A.5A.9) remains the starting point for all quantitative analyses of these processes, it is rare that it provides the complete picture. Site heterogeneity, ligand multivalence, ligand and acceptor self-association or isomerization, and the binding of ligands to linear arrays of sites are just a few of the deviations from simple binding behavior that have been discussed in this unit. A common thread in this discussion is the matching of a binding model to experimental results. It has not been the authors' intention to provide an exhaustive list of the variety of models that have so far been examined in the literature, but rather to illustrate an approach that involves the formulation of a model-dependent binding function and a testing of the experimental data against the derived equations.

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VOLUME 1			
Main TOC: 1-6	Main TOC: 1-6	Main table of contents	Reflects new material in Chaps. 3, 18, 19, and Appendices, and new Chapter 20
Ch 3:1-2	Ch 3:1-2	Chapter 3 contents	Reflects new *UNIT 3.7*
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—	3.7.1-3.7.10	Metabolic labeling with amino acids	New *UNIT 3.7*
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VOLUME 2			
—	16.0.1-16.0.2	Chapter 16 introduction	New introduction
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18.0.1-18.0.2	18.0.1-18.0.2	Chapter 18 introduction	Revision
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—	19.4.1-19.4.9	Detection of protein-protein interactions by coprecipitation	New *UNIT 19.4*
—	New Chapter 20 & tab (40 pages)	Quantitation of protein interactions	New Chapter 20
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—	A.1D.1-A.1D.8	Common conversion factors	New *APPENDIX 1D*
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CHAPTER 3
Detection and Assay Methods

INTRODUCTION

The success or failure of protein-centered projects can frequently be traced to the quality of the analytical procedures used to characterize the sample at different stages. Qualitative and quantitative analysis can aid in definition of the sample for the purpose of designing separations. A knowledge of the properties of the desired protein (e.g., whether it has a high aromatic amino acid content) can suggest methods of analysis that will help locate the desired protein in a complex mixture. Establishing the properties of an isolated protein creates benchmarks that future researchers can use to evaluate their protocols and final product. Accurate quantitation of the amount of protein at the beginning, middle, and end of a series of steps is the only valid way to evaluate the overall yield of a procedure. The observation of significant loss of protein following a particular purification procedure, without a substantial increase in the purity of the desired protein, would indicate that the procedure should be omitted or revised.

Several spectroscopic procedures for characterizing protein samples are described in *UNIT 3.1*. Measuring the absorbance of the aromatic amino acids in a protein at different wavelengths yields a very useful measure of protein concentration. This is nondestructive and requires very little sample or time. A more qualitative, but much more sensitive, evaluation is provided by fluorescence spectroscopy.

Another approach is amino acid analysis—qualitative analysis, to determine purity, and quantitative analysis, to provide concentrations—both of which are presented in *UNIT 3.2*. Procedures are also given for calculating amino acid ratios from primary analytical data. Significant advances that have improved the precision and sensitivity of amino acid analysis have reinvigorated this method, which had for some years been neglected.

A common method for enhancing the sensitivity of protein detection is to radiolabel the proteins, as detailed in *UNIT 3.3*. This approach may also be employed to help quantitate the binding of peptide to other molecules or for radioimmunoassays. Several procedures for iodination (introduction of ^{125}I or ^{131}I) are presented; the choice between them will depend on the sample type, the content of residues sensitive to oxidation, and the amino acids that are critical to function. Procedures for handling radioactive samples, for separating labeled from unlabeled peptides, and for iodination to equimolar or higher levels (using a mixture of radioactive and nonradioactive iodine isotopes) are also provided. When Tyr or His residues are absent, or cannot be iodinated because they are critical for function, alternative methods involving the incorporation of ^{14}C or ^{3}H may be employed instead. The methods described include labeling by acetylation at amino groups on lysine or at the amino terminus using the Bolton-Hunter reagent or acetic anhydride, by reductive alkylation, and by labeling at Lys residues with iodoacetic acid or iodoacetamide. Two synthetic methods, reduction of a peptide containing iodo-Tyr residues and incorporation of labeled amino acids, are also presented.

Measurement of the total protein content of a sample is discussed in *UNIT 3.4*. Three methods based on reduction of Cu^{2+} to Cu^{+} followed by chelation of the cuprous ion by various reagents (biuret, Hartree-Lowry, and bicinchinonic acid) are presented. In addition, a quantitative measure of total protein based on protein hydrolysis and the ninhydrin

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reaction is described. The use of UV absorption, Coomassie dye binding, and weight analysis to determine protein content complete the presentation. Important considerations for performing these assays, such as dealing with interfering substances, are detailed throughout the unit.

Some aspects involved in the design of enzyme assays are discussed in *UNIT 3.5*. Parameters that affect both enzyme and substrate are evaluated and suggestions to optimize the quantitation of enzymatic activity are enumerated. The proper concentration range for conducting initial velocity measurements is presented, and different methods of data presentation and analysis are discussed.

Tagging a protein with a group that permits facile detection is an excellent way, for example, to study the movement of a protein through internalization or compartmentalization. A variety of reagents are available for attaching biotin to functional groups or the surface of a protein (*UNIT 3.6*). Detection and localization can be achieved by the strong and specific binding of avidin or streptavidin. These reactions can also be used to label proteins at cell surfaces.

Another highly sensitive approach to the detection of proteins is the incorporation of radiolabeled amino acids into proteins. Integration of a particular radiolabeled amino acid (i.e., [^{35}S]D-methionine or [^{3}H]leucine) can be achieved using the normal cellular machinery (*UNIT 3.7*). Both pulse-labeling methods that permit study of cellular trafficking and long-term labeling to increase the detection of relatively rare proteins are described.

Analytical assay methods to quantitate total protein content by biological activity will be presented in future updates to this chapter.

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Metabolic Labeling with Amino Acids

Metabolic labeling techniques are used to study the biosynthesis, processing, intracellular transport, secretion, degradation and physical-chemical properties of proteins. In this unit, protocols are described for metabolically labeling mammalian cells with radiolabeled amino acids (Table 3.7.1). Cells labeled using these procedures are suitable for analysis by immunoprecipitation, characterization of cellular proteins, analysis of protein trafficking, and one- and two-dimensional gel electrophoresis (*UNITS 10.1-10.4*). The first protocols describe pulse-labeling (10 to 30 min) of mammalian cells in suspension with [^{35}S]methionine (see Basic Protocol), and necessary modifications for adherent mammalian cells (see Alternate Protocol 1). Alternate protocols are also presented for pulse-chase analysis (see Alternate Protocol 2) and long-term ("steady state") labeling (6 to 32 hr; see Alternate Protocol 3). This is followed by conditions for metabolic labeling of cells with radiolabeled amino acids other than [^{35}S]methionine (see Alternate Protocol 4). The degree of label incorporation can be determined by precipitation with trichloroacetic acid (TCA; see Support Protocol).

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) may require altered levels of CO_2 to maintain pH 7.4.

Table 3.7.1 Radiolabeled Amino Acids Used in Metabolic Labeling of Proteins

Amino acid[a]	Frequency (%)[b]	Radioisotope	Specific activity (Ci/mmol)	Comments[c]
Leucine	10.4	^{3}H	5-190	E
Serine	8.1	^{3}H	5-40	T, I
Glutamic acid	7.3	^{3}H	15-80	T, I
Lysine	7.0	^{3}H	40-110	E
Alanine	7.0	^{3}H	10-85	T
Valine	6.2	^{3}H	10-65	E
Glycine	5.7	^{3}H	10-60	T, I
Threonine	5.6	^{3}H	5-25	E
Arginine	5.0	^{3}H	30-70	E
Aspartic acid	4.9	^{3}H	10-50	T, I
Proline	4.9	^{3}H	15-130	—
Glutamine	4.5	^{3}H	20-60	T, I
Phenylalanine	4.5	^{3}H	15-140	E
Tyrosine	3.6	^{3}H	15-60	—
Asparagine[d]	3.5	—	—	—
Cysteine	3.4	^{35}S	>800	—
Isoleucine	2.9	^{3}H	30-140	E
Histidine	2.5	^{3}H	30-70	E
Methionine	1.8	^{35}S	>800	E
Tryptophan	1.3	^{3}H	20-30	E

[a]All amino acids in this table are in the L configuration.
[b]Frequency of amino acid residues in proteins, taken from Lathe (1985).
[c]E, essential amino acids; T, amino acids that are modified by transamination (Coligan et al., 1983); I, amino acids that are converted by cells to other amino acids. See Critical Parameters for more details.
[d]Asparagine is difficult to label (Coligan et al., 1983).

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SAFETY PRECAUTIONS FOR WORKING WITH ^{35}S-LABELED COMPOUNDS

When working with radioactive materials, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following guidelines provided by the local radiation safety officer (also see *APPENDIX 2B*).

Solutions containing ^{35}S-labeled compounds have been found to release volatile radioactive substances (Meisenhelder and Hunter, 1988). In addition to the usual safety practices followed when handling radioactive materials (see *APPENDIX 2B*), some extra precautions should be taken when using ^{35}S-labeled amino acids:

1. Vials containing ^{35}S-labeled compounds should always be handled in a designated fume hood equipped with an activated charcoal filter. This includes thawing the solution, opening the vial, and adding the radiolabeled amino acid to the labeling medium. Before opening, vials should be vented with a needle attached to a syringe packed with activated charcoal. Tissue culture hoods should not be used for this purpose, as they are likely to become contaminated.
2. Minimize exposure of ^{35}S-containing solutions to the air.
3. Use baths, incubators, and centrifuges designated for work with radioactive materials. Place a tray containing a layer of activated charcoal in the CO_2 incubator to reduce the amount of ^{35}S-labeled compounds released to the air during cell labeling. Alternatively, filters impregnated with activated carbon (β-Safe, Schleicher & Schuell) can be attached to the covers of tissue culture dishes.
4. Monitor areas used during labeling by conducting wipe tests.
5. Dispose of solid and liquid ^{35}S waste quickly and with appropriate precautions.

BASIC PROTOCOL

PULSE-LABELING OF CELLS IN SUSPENSION WITH [^{35}S]METHIONINE

Pulse-labeling of proteins is performed by incubating cells for short periods (≤30 min) in culture medium containing a radiolabeled amino acid. The conditions described below are optimized for labeling times of 10 to 30 min, but the same procedure can be used for periods up to 6 hr, although fewer cells and/or more labeling medium should be used for optimal results. Very short pulses (<10 min) may require special conditions (see Critical Parameters). For labeling times >6 hr, see Alternate Protocol 3. [^{35}S]methionine is the radiolabeled amino acid of choice for metabolic labeling of proteins because of its high specific activity (>800 Ci/mmol) and ease of detection. A potential disadvantage of [^{35}S]methionine is its low abundance (~1.8% of the average amino acid composition; see Table 3.7.1). For proteins that contain little (e.g., only one) or no methionine, other radiolabeled amino acids should be used (see Alternate Protocol 4).

Materials

[^{35}S]L-Methionine (>800 Ci/mmol) or ^{35}S-labeled protein hydrolyzate (>1000 Ci/mmol)
Pulse-labeling medium (see recipe), warmed to 37°C
Cell suspension (e.g., Jurkat, RBL, K562, BW5147, or T or B cell hybridomas), grown in a humidified 37°C, 5% CO_2 incubator or prepared from tissues (e.g., lymphocytes)
PBS (*APPENDIX 2E*), ice cold
Vacuum aspirator with trap for liquid radioactive waste

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Additional reagents and equipment for TCA precipitation (optional; see Support Protocol)

1. Thaw [^{35}S]methionine at room temperature and prepare a 0.1 to 0.2 mCi/ml working solution in prewarmed (37°C) pulse-labeling medium.

   CAUTION: *Volatile ^{35}S-containing compounds can be released during the labeling procedure (refer to Safety Precautions and APPENDIX 2B). Keep [^{35}S]methionine-containing medium in a tightly capped tube in a 37°C water bath until use. Do not let it sit >60 min at 37°C.*

2. Harvest 0.5–2 × 10^7 cells in suspension by centrifuging 5 min at 300 × *g*, room temperature.

3. Aspirate supernatant and resuspend cells by gently tapping the bottom of the tube. Add 10 ml prewarmed pulse-labeling medium. Mix by inversion of the tube and centrifuge 5 min at 300 × *g*, room temperature. Repeat this step once.

4. Resuspend cells at 5 × 10^6 cells/ml in prewarmed pulse-labeling medium and incubate 15 min in a 37°C water bath to deplete intracellular pools of methionine. Invert tube periodically to resuspend cells.

5. Centrifuge cells 5 min at 300 × *g*, room temperature, and aspirate supernatant.

6. Resuspend cells in 2 ml [^{35}S]methionine working solution (step 1). Cap tube tightly. Incubate cells 10 to 30 min (see Critical Parameters) in a 37°C water bath, resuspending frequently by gentle inversion of the tube.

   Alternatively, use a rotator placed in a 37°C incubator.

7. Centrifuge cells 5 min at 300 × *g*, 4°C, and aspirate supernatant. Resuspend with gentle swirling in 10 ml ice-cold PBS and repeat centrifugation.

   CAUTION: *The medium and wash are radioactive—follow applicable safety regulations for disposal.*

   Because of the short times and high concentrations of radiolabeled amino acids employed, pulse-labeling may not result in complete depletion of label from the medium. If this is the case, the labeling mixture can be reused. Collect the labeling medium containing [^{35}S]methionine or ^{35}S-labeled protein hydrolyzate and filter carefully through a 0.45-μm filter unit. Estimate the percentage of unincorporated radioactivity by scintillation counting aliquots of the labeling mixture before and after labeling the cells. Store the labeling mixture up to 2 months frozen at –20°C.

8. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

   Labeled cells are now ready for the desired processing and analysis. If cell pellets cannot be processed immediately, they can be kept on ice for a few hours or frozen at –80°C for several days. Thaw frozen cell pellets on ice before analysis. In most cases, freezing cells will not affect the biochemical properties of the proteins, but freezing and thawing cell extracts after solubilization with detergents (APPENDIX 1B) can cause dissociation of multisubunit complexes or proteolysis of labeled proteins.

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ALTERNATE PROTOCOL 1

PULSE-LABELING OF ADHERENT CELLS WITH [^{35}S]METHIONINE

Labeling of adherent cells is essentially the same as described for cells in suspension except that cells are labeled while attached to a dish. As with suspended cells, adherent cells are pulse-labeled 10 to 30 min (see Basic Protocol introduction). Alternatively, some adherent cells can be detached from plates by incubation for 10 min at 37°C with 10 mM EDTA in PBS (*APPENDIX 2E*) and labeled as a suspension (see Basic Protocol). This is particularly advantageous when labeling cells for pulse-chase experiments, because it simplifies handling of multiple samples and allows more uniform labeling of the cells.

Additional Materials (also see Basic Protocol)

Adherent cells (e.g., HeLa, NRK, M1, COS-1, CV-1, or fibroblasts or endothelial cells in primary culture)
100-mm tissue culture dishes

1. Grow adherent cells to 80% to 90% confluency in 100-mm tissue culture dishes.

   Depending on the cell type, a confluent 100-mm dish will contain 0.5–2 × 10^7 cells.

2. Prepare [^{35}S]methionine working solution (see Basic Protocol, step 1).

3. Aspirate culture medium from the dishes and wash twice by gently swirling with 10 ml prewarmed (37°C) pulse-labeling medium, aspirating medium after each wash.

4. Add 5 ml prewarmed pulse-labeling medium and incubate 15 min in a humidified 37°C, 5% CO_2 incubator to deplete intracellular pools of methionine.

5. Remove medium from cells, add 2 ml [^{35}S]methionine working solution (from step 2), and incubate 10 to 30 min in a CO_2 incubator.

   If necessary, as little as 1 ml of labeling medium per plate can be used. It is critical, however, that the plates sit on a perfectly horizontal surface during incubation to avoid drying of the cell monolayer.

6. Remove medium from cells. Wash once with 10 ml ice-cold PBS and remove PBS.

   CAUTION: *The medium and wash are radioactive—follow applicable safety regulations for disposal.*

   If significant detachment of cells occurs during labeling, it may be necessary to scrape the cells carefully in the [^{35}S]methionine-containing medium and transfer the suspension to a 15-ml centrifuge tube before centrifuging and washing with PBS.

7. Add 10 ml ice-cold PBS and scrape cells carefully with either a disposable plastic scraper or a rubber policeman.

8. Transfer the suspension to a 15-ml centrifuge tube, centrifuge 5 min at 300 × *g*, 4°C, and discard supernatant.

9. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

   Labeled cells are now ready for the desired processing and analysis (see Basic Protocol, step 8 annotation, if cells are not to be used immediately).

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PULSE-CHASE LABELING OF CELLS WITH [^{35}S]METHIONINE

ALTERNATE PROTOCOL 2

Pulse-chase protocols are used to analyze time-dependent processes, such as posttranslational modification, transport, secretion, or degradation of newly synthesized proteins. Cells in suspension or adherent cells are pulse-labeled with [^{35}S]methionine (see Basic Protocol and Alternate Protocol 1), after which they are incubated (chased) in complete medium containing excess unlabeled methionine, as described below.

Additional Materials (also see Basic Protocol and Alternate Protocol 1)

Chase medium (see recipe), 37°C

1. Prepare 0.5–2 × 10^7 cells per sample per time point, and pulse-label 10 to 30 min with 2 ml of 0.1 to 0.2 mCi/ml [^{35}S]methionine per 0.5–2 × 10^7 cells (see Basic Protocol, steps 1 to 6, or see Alternate Protocol 1, steps 1 to 5).
2. Remove the [^{35}S]methionine working solution, wash once with 10 ml of 37°C chase medium, and add 10 ml of 37°C chase medium.

   Rapid termination of the labeling reaction can be achieved by adding two times the volume of chase medium containing excess unlabeled methionine (15 mg/liter) directly to the labeling mixture.

   Final concentration of cells in suspension should be 2 × 10^6 cells/ml for chases ≤2 hr, or 0.5 × 10^6 cells/ml for chases >2 hr. For adherent cells, add 10 ml/100-mm dish.
3. Incubate for the desired time at 37°C. Incubate cell suspensions with rotation in tightly capped tubes. Incubate adherent cells in a CO_2 incubator.

4a. *For cells in suspension:* Collect cells by centrifuging 5 min at 300 × *g*, 4°C.

   The supernatant can be discarded or can be collected for analysis of proteins that are secreted or shed into the medium.

4b. *For adherent cells:* Scrape off adherent cells and transfer to 15-ml centrifuge tubes. Centrifuge 5 min at 300 × *g*, 4°C, and either save or discard the supernatant as in step 4a.

5. *Optional:* Determine amount of label incorporation by trichloroacetic acid (TCA) precipitation (see Support Protocol 1).

   Labeled cells are now ready for the desired processing and analysis (see Basic Protocol, step 8 annotation, if cells are not to be used immediately).

LONG-TERM LABELING OF CELLS WITH [^{35}S]METHIONINE

ALTERNATE PROTOCOL 3

Long-term labeling refers to continuous metabolic labeling of cells for periods of 6 to 32 hr. Long-term labeling is particularly advantageous when studying proteins that are synthesized at low rates. It is also used to accumulate mature labeled proteins, rather than biosynthetic precursors, for characterization of their properties. Steady-state labeling is a form of long-term labeling in which cells are incubated with the radiolabeled amino acid until the rates of synthesis and degradation of the radiolabeled proteins are equal. Steady-state labeling allows calculation of the stoichiometry of subunits within a protein complex, provided that the primary structure of the subunits is known. In these procedures, unlabeled methionine is added to the medium to maintain cell viability and to sustain incorporation of label for the duration of the experiment. The amount of unlabeled methionine added depends on factors such as the length of the labeling period and the cell density. Media containing between 5% and 20% the normal amount of unlabeled methionine are generally used. The conditions described below are suitable for overnight (~16 hr) labeling of cells in suspension or adherent cells.

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Additional Materials (also see Basic Protocol and Alternate Protocol 1)

Long-term labeling medium (see recipe), warmed to 37°C
75-cm^2 tissue culture flask

1. Prepare a 0.02 to 0.1 mCi/ml [^{35}S]methionine working solution in prewarmed (37°C) long-term labeling medium (see Basic Protocol, step 1).

For cell suspensions:

2a. Prepare and wash cells once with prewarmed long-term labeling medium (see Basic Protocol, steps 2 and 3).

3a. Resuspend 0.5–2 × 10^7 cells in 25 ml of 0.02 to 0.1 mCi/ml [^{35}S]methionine working solution and transfer to a 75-cm^2 tissue culture flask.

For adherent cells:

2b. Grow 0.5–2 × 10^7 adherent cells in a 75-cm^2 tissue culture flask (80% to 90% confluency) in a CO_2 incubator. Wash once with prewarmed long-term labeling medium (see Alternate Protocol 1, step 3).

3b. Add 25 ml of 0.02 to 0.1 mCi/ml [^{35}S]methionine working solution to each 75-cm^2 flask.

4. Tighten caps to prevent release of volatile ^{35}S-labeled compounds. Incubate 16 hr in a CO_2 incubator.

5. Wash cells and determine incorporation (see Basic Protocol, steps 7 and 8, or Alternate Protocol 1, steps 6 to 9).

Labeled cells are now ready for the desired processing and analysis (see Basic Protocol, step 8 annotation, if cells are not to be used immediately).

ALTERNATE PROTOCOL 4

METABOLIC LABELING WITH OTHER RADIOLABELED AMINO ACIDS

In some instances, it may be necessary to label proteins with radiolabeled amino acids other than [^{35}S]methionine—e.g., when the proteins have a low methionine content or have no methionine residues at all. In these cases, the best choices are [^{35}S]cysteine or [^{3}H]leucine.

Cysteine residues are more abundant in proteins than methionine residues (3.4% versus 1.8%; Table 3.7.1), although cysteine is less stable. Formulations of [^{35}S]cysteine that have high specific activity (>800 Ci/mmol) can be obtained from several companies. Leucine has the advantage of being the most frequent amino acid in proteins (10.4%; Table 3.7.1) and specific activities of [^{3}H]leucine are the highest among ^{3}H-labeled amino acids (up to 190 Ci/mmol). If proteins are known to be rich in a particular amino acid or if special methods are to be performed (e.g., radiochemical sequencing or multiple labeling), ^{3}H-labeled amino acids other than [^{3}H]leucine can be used.

Label cells with these amino acids as described for [^{35}S]methionine in the previous protocols. Substitute [^{35}S]cysteine or ^{3}H-labeled amino acids in the labeling medium. Use labeling medium lacking cysteine or any other respective amino acid.

CAUTION: Solutions containing [^{35}S]cysteine release volatile ^{35}S-labeled compounds (refer to Safety Precautions).

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TCA PRECIPITATION TO DETERMINE LABEL INCORPORATION

In metabolic labeling experiments, it is often useful to monitor the incorporation of radioactivity into total cellular proteins. This can easily be achieved by precipitation with trichloroacetic acid (TCA) using BSA as a carrier protein. This protocol can be used at the end of the labeling procedures in each protocol.

Materials

Labeled cell suspension (see Basic Protocol or Alternate Protocols 1 to 4)
BSA/NaN_3: 1 mg/ml BSA containing 0.02% (w/v) sodium azide (NaN_3)
10% (w/v) TCA solution (see recipe), ice cold
Ethanol

Filtration apparatus attached to a vacuum line
2.5-cm glass microfiber filter disks (Whatman GF/C)

CAUTION: TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.

1. Add 10 to 20 µl of a labeled cell suspension to 0.1 ml BSA/NaN_3. Place on ice.
2. Add 1 ml ice-cold 10% (w/v) TCA solution. Vortex vigorously and incubate 30 min on ice.
3. Filter the suspension onto 2.5-cm glass microfiber filter disks in a filtration apparatus under vacuum.
4. Wash the disks twice with 5 ml ice-cold 10% (w/v) TCA solution and twice with ethanol. Air dry 30 min.

   CAUTION: *The wash fluids should be handled as mixed chemical/radioactive waste—follow applicable safety regulations for disposal (see APPENDIX 2B).*

5. Spot the same volume of the radiolabeled cell suspension used in step 1 (10 to 20 µl) on a glass microfiber disk. Air dry.

   This disk will be used to measure the total amount of radiolabeled amino acid in the cell suspension.

6. Transfer disks from steps 4 and 5 to 20-ml scintillation vials, add 5 ml scintillation fluid, and measure the radioactivity in a scintillation counter.
7. Calculate the ratio of TCA-precipitable label to total radioactivity (i.e., the ratio of sample radioactivity in step 4 to the total radioactivity in step 5).

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

NOTE: Use sterile tissue culture technique to prepare these reagents.

Chase medium

Pulse-labeling medium (see recipe) containing 15 mg/liter unlabeled methionine or equivalent excess amount of other amino acid used for radiolabeling. Store up to 2 weeks at 4°C.

Long-term labeling medium (90% methionine-free medium)

Mix 9 vol pulse-labeling medium (see recipe), lacking methionine or other appropriate amino acid, with 1 vol chase medium (see recipe) containing the same amino acid. Store up to 2 weeks at 4°C.

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Pulse-labeling medium

Use supplemented Dulbecco's modified Eagle medium (DMEM; see *APPENDIX 3C* but omit nonessential amino acids) or RPMI 1640, each lacking methionine or other specific amino acid, but containing 10% (v/v) FBS (dialyzed overnight against saline solution to remove unlabeled amino acids). Add 25 mM HEPES buffer (with pH adjusted to 7.4 with NaOH). Store up to 2 weeks at 4°C.

The amino acid used to label cells is omitted from this medium (e.g., if [^{35}S]methionine is employed, methionine-free DMEM or RPMI 1640 must be used). Specific amino acid–free media can be obtained from several tissue culture medium suppliers, or can be prepared (see APPENDIX 3C) from amino acid–free medium by adding individual amino acid components, omitting the one that will be used to label. A kit for the preparation of such media is available (Select-Amine from Life Technologies).

Trichloroacetic acid (TCA) solution, 10% (w/v)

Prepare a 100% (w/v) TCA stock solution by dissolving the entire contents of a newly opened TCA bottle in water (e.g., dissolve the contents of a 500-g bottle of TCA in sufficient water to yield a final volume of 500 ml). Store up to 1 year at 4°C. Prepare 10% (w/v) TCA by dilution and store up to 3 months at 4°C.

CAUTION: *TCA is extremely caustic. Protect eyes and avoid contact with skin when preparing and handling TCA solutions.*

COMMENTARY

Background Information

Metabolic labeling of cellular proteins is achieved by placing cells in a nutritional medium containing all components necessary for growth of cells in culture, except for one amino acid that is substituted by its radiolabeled form. The radiolabeled amino acids are transported across the plasma membrane by carrier-mediated systems and, once in the cytosol, are loaded onto tRNA molecules before being incorporated into newly synthesized proteins.

Because metabolic labeling techniques use the metabolic machinery of the cell to incorporate radiolabeled amino acids, there are limitations on the type of radiolabeled amino acids that can be employed. The list of potential precursors is restricted to L-amino acids normally found in proteins, in which one or more atoms are substituted by a radioisotope (Table 3.7.1). Sulfuric amino acids, such as methionine and cysteine, are conveniently labeled with ^{35}S. Most other amino acids can be obtained labeled with ^{3}H.

Critical Parameters

The protocols in this unit should be considered as models from which more specific methods can be designed. The conditions described here have been optimized for radiolabeling proteins that are expressed at low to moderate levels (10^4 to 10^5 copies per cell) and assume that the labeled cells will be used for immunoprecipitation (*UNIT 3.8*). This level of expression is characteristic of most endogenous membrane proteins, lumenal organellar proteins, signal transduction proteins, and transcription factors. More abundant proteins, such as cytoskeletal proteins or proteins expressed by infection or transfection, can be labeled with less radiolabeled amino acid and/or fewer cells. The choice of a particular labeling protocol and its modification will be aided by careful consideration of a number of parameters that influence the incorporation of radiolabeled amino acids into proteins, as discussed below.

Selection of amino acid label

Purified [^{35}S]methionine of high specific activity (>800 Ci/mmol) can be obtained from several suppliers of radiolabeled amino acids. Protein hydrolyzates of *Escherichia coli* grown in the presence of $[^{35}SO_4]^{2-}$ (e.g., Tran^{35}S-label from ICN Biomedicals or Expre^{35}S^{35}S from NEN Life Sciences) can be used as substitutes for [^{35}S]methionine in metabolic labeling techniques. These preparations contain ^{35}S distributed among several different compounds. In a typical batch, ~70% of the radioactivity will be present in the form of [^{35}S]methionine and ~15% as [^{35}S]cysteine; the remainder is other ^{35}S-labeled compounds. Labeling with these radioactive protein hydrolyzates in methionine-free medium will result in incorporation of label only in methionine residues; use of

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methionine- and cysteine-free medium will result in labeling of both methionine and cysteine residues.

Purified [^{35}S]methionine should be used whenever certainty of labeling with only [^{35}S]methionine is required. Another reason for using purified [^{35}S]methionine is that it tends to be more stable and emit less volatile decomposition products than ^{35}S-labeled protein hydrolyzates. In most cases, however, the relatively inexpensive radioactive protein hydrolyzates (about one-third the cost of [^{35}S]methionine) can be used instead of purified [^{35}S]methionine. [^{35}S]methionine preparations should be stored frozen at −80°C. Under these conditions, they are stable for at least 1 month; the half-life of ^{35}S is 88 days.

For proteins with one or no methionine residues but several cysteine residues, labeling with [^{35}S]cysteine (available with specific activities >800 Ci/mmol) is a good option. ^{35}S-labeled protein hydrolyzates are not good sources of [^{35}S]cysteine for radiolabeling because only ~15% of the ^{35}S-labeled compounds are [^{35}S]cysteine.

Purified [^{3}H]leucine (available with specific activities of up to 190 Ci/mmol) is a good alternative to ^{35}S-labeled amino acids. The half-life of ^{3}H is ~12 years. The specific activities of other ^{3}H-labeled amino acids range between 5 and 140 Ci/mmol (Table 3.7.1). Several problems can arise when using certain ^{3}H-labeled amino acids due to their participation in metabolic pathways. The following problems must be considered when using tritiated amino acids for metabolic labeling of proteins.

Nonessential versus essential amino acids. Cells are able to synthesize nonessential amino acids from other compounds. If the radiolabeled amino acids used are nonessential, their specific activity will be reduced by dilution with the endogenously synthesized amino acids. Therefore, essential amino acids (E in Table 3.7.1) should be favored for metabolic labeling.

Transamination. The α-amino groups of many amino acids can be removed in reactions catalyzed by transaminases (T in Table 3.7.1). Deamination of the radiolabeled amino acids can be prevented by addition of the transaminase inhibitor (aminooxy)acetic acid during starvation and labeling of the cells (Coligan et al., 1983).

Interconversion. Certain radiolabeled amino acids can be converted by cells into other amino acids (I in Table 3.7.1). This problem is of particular importance when one is attempting to determine the amino acid composition or sequence of radiolabeled proteins.

Labeling time

The experimental purpose, the turnover rate of the protein of interest, and the viability of the cells should all be considered when determining the length of time for labeling. If the purpose of the experiment is to identify or characterize a protein precursor, pulse-labeling (10 to 30 min) must be used (see Basic Protocol and Alternate Protocol 1). If the protein of interest has a low turnover rate or if it is necessary to accumulate a labeled mature product, a protocol for long-term labeling (6 to 32 hr) is more appropriate (see Alternate Protocol 3). If the biosynthesis, post-translational modification, intracellular transport, or fate of newly synthesized proteins is being analyzed, a pulse-chase protocol should be used (see Alternate Protocol 2).

The turnover rate of the protein of interest is an important parameter to be considered when determining the labeling time. Proteins with a high turnover rate are optimally labeled for short times. Longer times will result in increased labeling of other cellular proteins, causing a decrease in the relative abundance of the labeled protein of interest in the cell lysate. This could result in increased detection of nonspecific proteins in immunoprecipitates (see *UNIT 3.8*). Conversely, proteins that turn over slowly should be labeled for longer times.

Incorporation of radiolabeled amino acids into proteins is directly proportional to the labeling time for a certain period, after which it tends to plateau. When all the limiting amino acid is consumed, protein synthesis ceases. The length of the initial phase of linear incorporation will vary with the concentration of the labeling amino acid and the density and metabolic activity of the cells.

Concentration and specific activity of the radiolabeled amino acid

Because radiolabeled amino acids are used in limiting amounts, their incorporation into proteins is directly proportional to their concentration in the labeling medium. If very short pulses are required, the concentration of labeled amino acid can be increased to compensate for the shorter labeling time. The incorporation of radiolabeled amino acids is also directly proportional to their specific activity. Addition of unlabeled amino acids, as is required in long-term labeling protocols, will result in reduced incorporation over short periods.

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Cell density

At low cell densities, the amount of labeled protein synthesized is directly proportional to the cell concentration. At high densities, however, incorporation will increase nonlinearly. Very concentrated cell samples (>5 × 10^7 cells/ml) can only be labeled for short periods (≤5 min) as rapid loss of metabolic activity and cell viability will occur due to acidification of the medium and accumulation of toxic metabolites.

Conditions for very short pulses

Very short pulses (2 to 10 min) are required to study post-translational modifications that occur shortly after synthesis. These include folding, disulfide bond formation, and early carbohydrate modifications of newly synthesized proteins. Proteins that are rapidly degraded after synthesis are also best studied with very short pulses. The protocol for very short pulses is similar to the Basic Protocol for pulse-labeling, with the following modifications: (1) the pulse-labeling medium contains higher concentration of radiolabeled amino acid (up to 1 mCi/ml), (2) labeling is stopped by addition of ice-cold PBS containing 20 mM freshly prepared *N*-ethylmaleimide (NEM) to prevent oxidation of free sulfhydryls, and (3) if a chase is necessary, 1 mM cycloheximide is added to the chase medium to quickly stop the elongation of nascent polypeptide chains. For additional details on very short pulses, see Braakman et al. (1991).

Temperature and pH

Unless otherwise required for special conditions (e.g., heat shock, temperature-sensitive mutants), metabolic labeling should be performed at 37°C. For this reason, it is important that the labeling medium be warmed to 37°C before adding it to the cells. A dramatic reduction in incorporation occurs at room temperature. It is also important that the pH of the labeling medium be ~7.4.

Anticipated Results

A typical incorporation of labeled amino acid precursor after a 30-min pulse under the conditions described in this unit (see Basic Protocol and Alternate Protocols 1 and 2) is 5% to 20%. In the case of long-term labeling, the incorporation typically reaches 30% to 60%. If the labeled cells are used for immunoprecipitation (*UNIT 3.8*), followed by SDS-PAGE (*UNIT 10.1*) and autoradiography (*UNIT 10.11*), specific bands should be visible within 2 hr to 2 months of exposure.

Time Considerations

It takes 1 to 2 hr to prepare cells and materials for labeling. The actual labeling time depends upon the protocol chosen—pulse-labeling takes 10 to 30 min and long-term labeling takes 6 to 32 hr. Washing and processing cells may take an additional 1.5 hr.

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Lathe, R. 1985. Synthetic oligonucleotide probes deduced from amino acid sequence data. Theoretical and practical considerations. *J. Mol. Biol.* 183:1-12.

Meisenhelder, J. and Hunter, T. 1988. Radioactive protein labelling techniques. *Nature* 335:120.

Key Reference

Coligan et al., 1983. See above.

Contains a detailed description of conditions used to metabolically label proteins with different radiolabeled amino acids.

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5. Remove 2-µl aliquots of the supernatants and add 28 µl SDS-PAGE sample buffer. Analyze the samples by SDS-PAGE (*UNIT 10.1*) to determine the heat stability of the fusion protein and the minimum time of heat treatment required to obtain a good purification.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Casamino Acids (CAA), 2% (w/v)

20 g Casamino Acids (Difco-certified)
H_2O to 1 liter
Autoclave or filter sterilize through a 0.45-µm filter
Store ≤2 months at room temperature

Do not use technical-grade Casamino Acids because it has a higher NaCl content.

CAA/glycerol/ampicillin 100 medium

800 ml 2% (w/v) Casamino Acids (see recipe; 1.6% final)
100 ml 10× M9 salts (see recipe; 1× final)
100 ml 10% (v/v) glycerol (sterile; 1% final)
1 ml 1 M $MgSO_4$ (sterile; 1 mM final)
0.1 ml 1 M $CaCl_2$ (sterile; 0.1 mM final)
1 ml 2% (w/v) vitamin B1 (sterile; 0.002% final)
10 ml 10 mg/ml ampicillin (sterile; 100 µg/ml final)
Prepare fresh

IMC medium

200 ml 2% (w/v) Casamino Acids (see recipe; 0.4% final)
100 ml 10× M9 salts (see recipe; 1× final)
40 ml 20% (w/v) glucose (sterile; 0.5% final)
1 ml 1 M $MgSO_4$ (sterile; 1 mM final)
0.1 ml 1 M $CaCl_2$ (sterile; 0.1 mM final)
1 ml 2% (w/v) vitamin B1 (sterile; 0.002% final)
658 ml glass-distilled H_2O (sterile)
10 ml 10 mg/ml ampicillin (sterile; optional; 100 µg/ml final)
Use fresh

IMC plates

15 g agar [Difco; 1.5% (w/v)]
4 g Casamino Acids [Difco-certified; 0.4% (w/v)]
858 ml glass-distilled H_2O (sterile)
Autoclave 30 min
Cool in a 50°C water bath
100 ml 10× M9 salts (see recipe; 1× final)
40 ml 20% (w/v) glucose (sterile; 0.5% final)
1 ml 1 M $MgSO_4$ (sterile; 1 mM final)
0.1 ml 1 M $CaCl_2$ (sterile; 0.1 mM final)
1 ml 2% (w/v) vitamin B1 (sterile; 0.002% final)
10 ml 10 mg/ml ampicillin (sterile; optional; 100 µg/ml final)
Mix well and pour into petri plates
Store ≤1 month at 4°C

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M9 salts, 10×

60 g Na_2HPO_4 (0.42 M)
30 g KH_2PO_4 (0.24 M)
5 g NaCl (0.09 M)
10 g NH_4Cl (0.19 M)
H_2O to 1 liter
Adjust pH to 7.4 with NaOH
Autoclave or filter sterilize through a 0.45-μm filter
Store ≤6 months at room temperature

SDS-PAGE sample buffer

15% (v/v) glycerol
0.125 M Tris·Cl, pH 6.8 (*APPENDIX 2E*)
5 mM Na_2EDTA
2% (w/v) SDS
0.1% (w/v) bromphenol blue
1% (v/v) 2-mercaptoethanol (2-ME; add immediately before use)
Store indefinitely at room temperature

Tryptophan, 10 mg/ml

Heat 500 ml glass-distilled H_2O to 80°C. Stir in 5 g L-tryptophan until dissolved. Filter sterilize the solution through a 0.45 μm filter and store ≤6 months in the dark at 4°C.

COMMENTARY

Background Information

Two significant problems plague researchers who hope to express heterologous proteins in *Escherichia coli*: inefficient initiation of translation of many eukaryotic mRNA sequences on bacterial ribosomes (Stormo et al., 1982), and proteins that often form insoluble aggregates, called inclusion bodies, that are composed of misfolded or denatured proteins (Mitraki and King, 1989). Although successful protocols for refolding eukaryotic proteins from inclusion bodies can be developed, the process is always uncertain and usually time-consuming; in most instances it is preferable to prevent inclusion-body formation in the first place. The use of *trxA* fusions provides a solution to both problems.

Inefficient initiation of translation of eukaryotic messages in *E. coli* can often be improved by modifying sequences at the 5′ end of the gene. A more reliable technique that avoids the problem entirely is to use a gene-fusion strategy in which the gene of interest is linked in-frame to the 3′ end of a highly translated partner gene. In this case protein synthesis always initiates on the same efficiently translated fusion-partner mRNA, thus ensuring high-level expression. Some earlier gene-fusion expression systems, for example the *trpE* and *lacZ* systems, offer very reliable ways of producing large quantities of any desired eukaryotic protein. However, these gene-fusion systems still suffer from the pervasive inclusion-body problem. They are thus mainly useful for the production of antigens, rather than correctly folded, biologically active proteins. More recently the maltose binding protein (MBP; Riggs, 1994; *UNIT 5.1*) and glutathione-*S*-transferase (GST) gene fusion expression systems (see *UNIT 6.6*) have proven more successful in producing soluble fusion proteins; these systems retain the translation advantage of the earlier fusion systems. Apart from the obvious advantages in making a correctly folded product, the synthesis of soluble fusion proteins also allows for the development of generic purification schemes based on some unique property of the fusion partner.

Why would any particular eukaryotic protein produced in the *E. coli* cytoplasm be more soluble when it is linked to a fusion partner than it would be by itself? It is likely that physical properties of the fusion-partner protein are important, with efficient self-folding and high solubility being useful in this role. It is possible that some good fusion partners (proteins that fold efficiently and are highly soluble), by virtue of their desirable physical qualities, are able to keep folding intermediates of linked heterologous proteins in solution long enough for

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CHARACTERIZATION OF RECOMBINANT PROTEINS

7

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CHAPTER 10
Electrophoresis

INTRODUCTION

Electrophoresis of protein samples in polyacrylamide gels is an indispensable analytical and, in some cases, preparative tool for the protein scientist. Electrophoresis can be used to separate and compare complex protein mixtures, evaluate purity of a protein during the course of its isolation, and provide estimates of physical characteristics such as subunit composition, isoelectric point, size, and charge.

Each type of electrophoretic separation can be conducted in a variety of gel sizes ranging from microgels (e.g., Phast gels from Hoefer Pharmacia) only slightly larger than a postage stamp to giant gels much larger than this page (Garrels, 1979; Young et al., 1983). In general, the time required to electrophorese, stain, and destain small gels is very short, and a minimal amount of sample is consumed. In contrast, larger gels consume more reagents, sample, and time, but provide increased resolution. Small gels are therefore recommended for rapid screening, and larger gels are indicated when maximum resolution is required, as in analysis of complex mixtures or samples containing very similar components.

The most common one-dimensional gel methods utilize the detergent sodium dodecyl sulfate (SDS) to solubilize, denature, and impart a strong negative charge to proteins (*UNIT 10.1*). Although many one-dimensional SDS gel methods have been published over the past 30 years, the single most widely used method is that initially described by Laemmli (1970). SDS-based gel separations are very robust as most, but not all, proteins are readily solubilized in SDS solutions. Most proteins bind a uniform amount of SDS per microgram of protein, which imparts a uniform charge density per unit mass to provide a separation based on the mass of the polypeptide chain.

An alternative one-dimensional separation methodology is based on the protein's isoelectric point and is usually performed in the presence of denaturants such as urea (*UNIT 10.2*). Like one-dimensional SDS gel separations, this denaturing isoelectric focusing method is capable of separating a large number of components in a single dimension. A third one-dimensional method uses native conditions to separate proteins based on intrinsic charge (*UNIT 10.3*). Although this method has less resolving power than the previous two methods, it can be particularly useful for associating a specific electrophoretically separated component with a biological activity, since no denaturants are used.

Two-dimensional electrophoresis (*UNIT 10.4*) involves the orthogonal combination of two different electrophoretic methods. Any two electrophoresis techniques can be combined in this manner to produce useful separations. The most common two-dimensional approach combines two individually high-resolution methods, consisting of isoelectric focusing (*UNIT 10.2*) followed by SDS gel electrophoresis (*UNIT 10.1*). Each of these methods is individually capable of resolving up to about 100 protein bands; when the two are combined, more than 1,000 proteins can be separated on a single two-dimensional gel (O'Farrell, 1975; Garrels, 1979). Hence this method can be used to analyze and compare complex protein mixtures, including whole-cell or tissue extracts. One important application of two-dimensional gels is the systematic analysis and quantitative comparison of whole-cell or tissue extracts for the study of the proteome, or complete protein profile, of

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the cell or tissue. In recent years a growing emphasis has been focused on such studies, particularly for organisms whose complete genome sequence has been determined. Other applications of two-dimensional electrophoresis include assessment of the purity and homogeneity of a purified protein, including recombinant proteins (Chapter 7), and evaluation of some post-translational modifications (Chapters 12 and 13).

After electrophoretic separation, proteins are usually detected using general protein staining methods, such as Coomassie blue staining or the more sensitive silver staining. These staining methods "fix" the proteins in the gel matrix by either chemical cross-linking or denaturation to prevent subsequent diffusion of the protein bands (*UNIT 10.5*). When proteins are to be eluted or extracted from the gels for subsequent analyses, however, fixation-based stains should usually be avoided. In these cases, methods for protein detection without fixation should be used (*UNIT 10.6*) when it is necessary to stain a gel to locate the protein of interest.

A useful method of isolating proteins from gels for subsequent analysis involves electroblotting onto an inert support, such as polyvinylidene difluoride (PVDF) membranes (*UNIT 10.7*). As a result of the versatility of PVDF membranes, electroblotting has largely replaced electroelution as the method of choice for isolating proteins from most types of polyacrylamide gels (LeGendre and Matsudaira, 1988). PVDF membranes have very high protein binding capacities, good handling characteristics when either dry or wet, and are highly chemically inert. Hence they are compatible with many methods for subsequent analysis, including amino acid analysis, in situ chemical modification, in situ protease digestion, and N-terminal sequence analysis (Chapter 11). A wide range of general protein stains exist that are compatible with most blotting membranes (*UNIT 10.8*). Alternatively, radiolabeled proteins can be detected by autoradiography either in the gel or after transfer to a PVDF membrane (*UNIT 10.11*). The latter is normally preferable; as long as the proteins of interest are electrotransferred in high yield, autoradiography signals of weak β emitters such as ^{14}C or ^{35}S are usually higher on electroblotted membranes than in dried gels (even when fluorophores are impregnated in the gel), because the protein is primarily located on the surface of the membrane that was in contact with the gel during electroblotting. Historically, autoradiography was performed using medical X-ray films. More recently, a relatively wide range of commercially available autoradiography films and exposure methods have been developed that provide superior contrast and higher sensitivity for specific isotopes (*UNIT 10.11*).

The dimensional stability and chemical resistance of PVDF membranes provides an optimal medium for combining multiple detection methods, either sequentially on the same membrane, or on parallel lanes that can be reassembled after different detection methods have been performed. For example, sets of lanes containing replicate samples can be run on a single gel separated by prestained standards, which are used as guides to cut the resulting membrane. Each lane set can be subjected to different detection methods such as protein staining and immunoblotting with different antibodies (*UNIT 10.10*). The analyzed membranes can then be precisely reassembled to determine the relationships between bands detected by different methods. As there is no shrinkage or swelling of the membrane, this approach is particularly valuable for detecting slight differences in migration between components detected by different methods.

An approach that has recently emerged as a useful analytical method for separating peptides and, to a more limited extent, proteins is capillary electrophoresis (CE; *UNIT 10.9*). CE protocols have also been developed for preparative peptide isolation in adequate amounts for subsequent high sensitivity measurements such as micro-sequencing (Chapter 11) and mass spectrometry (Chapter 16). CE employs a fused-silica capillary column that may contain either free solution or a fluid matrix. One of CE's limitations for protein

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and peptide separations is the tendency of these molecules to interact with silanol groups on the capillary tubing surface. As described in *UNIT 10.9*, these interactions can be minimized through the use of coated capillaries, a separation pH several units above the protein pI, or buffer additives that minimize interactions with the silica walls. An interesting alternative approach is separation of proteins and peptides using capillary zone electrophoresis (CZE) in acidic amphoteric buffers (*UNIT 10.13*). The low conductivity of these buffers permits higher voltages to be used, resulting in faster separations, and the low pH minimizes ionization of silanol groups on underivatized capillaries. In this mode, nearly all proteins and peptides have strong positive charges and high mobilities. In contrast to the relatively simple equipment requirements of polyacrylamide-based electrophoretic methods, commercial CE instruments are similar in complexity and expense to high-performance liquid chromatography (HPLC) instruments, due to the necessity for a high-voltage supply, a high-performance cooling system, and a very sensitive detector. Because CE separates components by charge rather than by hydrophobicity as in reversed-phase HPLC, it is a highly useful orthogonal separation method that is frequently used to complement HPLC peptide separation strategies. Compared with polyacrylamide methods, CE offers much higher resolution, flexibility, sensitivity, and speed for separating peptides (<6 kDa), whereas polyacrylamide methods are more generally applicable for protein separations.

Documentation of gel electrophoresis results for either archival or publication purposes historically used primarily silver halide–based photography. In recent years, the availability of economical hardware and software for capturing, storing, and analyzing digital images has led to the replacement of photography by digital analysis as the preferred documentation method (*UNIT 10.12*). Advantages of digital-based methods include more convenient storage and retrieval, inexpensive and rapid production of backup or additional copies, facile manipulation such as resizing and inserting into reports, and rapid data distribution to colleagues over the Internet. Digital processing of electrophoretic results is particularly essential for comparing large numbers of gels, developing databases of gel patterns and related information, and performing quantitative comparisons such as proteome studies utilizing two-dimensional gels.

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CHAPTER 16
Mass Spectrometry

INTRODUCTION

From the point of view of protein scientists, in the course of the 1990s mass spectrometry (MS) has developed from a fairly exotic technique practiced in only a handful of laboratories into a routine, essential tool for protein characterization. MS is complementary to other protein and peptide analysis methods such as protein sequencing, amino acid analysis, and high-performance liquid chromatography (HPLC) peptide mapping (Chapter 11). It is particularly useful for identifying post-translational modifications (Chapters 12 to 14), which are often quite difficult to distinguish by other methods. To some extent, MS is beginning to replace alternative methods such as Edman sequencing as continuing advances in instruments, software, and user-friendliness improve the sensitivity and utility of MS methods.

A detailed discussion of peptide and protein MS analysis, including sample ionization methods, basic principles and parameters, and data interpretation, is presented in *UNIT 16.1*. The unit also lists numerous literature references that should be especially useful to scientists seeking more detailed information on specific MS-related issues.

A key advance in the application of MS for routine analysis of peptides and proteins has been the development of commercial instruments that employ matrix-assisted laser desorption/ionization (MALDI) techniques to transfer peptides and proteins efficiently into the gas phase for time-of-flight based mass measurements. This technique has multiple advantages compared with older techniques, including the instruments' relatively low cost and ease of operation, relatively good tolerance of nonvolatile buffer components, and ability to analyze heterogeneous samples as mixtures, since the ionization method predominantly produces a single charged species per sample component. A general discussion of MALDI-MS analysis of peptides is presented in *UNIT 16.2*, and *UNIT 16.3* provides specific protocols for analysis of both peptides and proteins, with emphasis on sample preparation.

A frequent research question in biology is the correlation of observed function with the proteins responsible. MS-based methods have emerged as the most sensitive, rapid, and economical method for identifying proteins. The simplest approach is MALDI-MS fingerprint mapping following in-gel protease digestion of the protein of interest (*UNIT 16.4*). The observed peptide masses are used to search sequence databases over the Internet (*UNIT 16.5*) using one of several alternative algorithms. This method works best when the protein of interest is expected to be found in an available sequence database—i.e., when the complete genome of the species from which the protein was isolated has been sequenced. When the database contains only related, but nonidentical, proteins, or when the results of the fingerprint mapping search are ambiguous, partial sequence information is generally required to identify homologous proteins or to search expressed-sequence tag (EST) databases. Such partial sequence information can be obtained on a MALDI spectrometer either using fragmentation induced within the mass analyzer (*UNIT 16.6*) or via exopeptidase digestion of samples on the MS sample target (*UNIT 16.7*).

The other common mass analysis method for biomolecules is electrospray ionization (ESI)-MS (*UNIT 16.1*). In recent years, the cost of ESI-based instruments has decreased and

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they have become more sensitive and user friendly. Triple-quadrupole or ion-trap instruments with ESI interfaces are particularly well suited to tandem mass spectrometry (MS-MS) experiments, in which an initial mass measurement is followed by fragmentation of a target peptide and reanalysis of fragment mass spectra. ESI-MS is much less tolerant of salts and buffers than MALDI-MS, and samples are typically introduced by interfacing an HPLC instrument using a reversed-phase column on the front end of the mass analyzer. This serves both to clean up the sample and to separate components prior to introduction of sample into the mass analyzer. When capillary or nanocapillary HPLC columns are used without stream splitting, very high sensitivities can be obtained. Future units will detail the use of ESI instruments for protein and peptide characterization, and protein identifications either using MS-MS data directly or using de novo sequence interpretation of MS-MS data.

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PREPARATION AND HANDLING OF PEPTIDES

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CHAPTER 18
Preparation and Handling of Peptides

INTRODUCTION

An important component of the armamentarium of modern protein scientists is the ability to design and construct peptides of almost any sequence. Peptides may be employed for a wide range of applications, including as substrates for kinases, proteases, or glycosidases, or as antigens to produce antisera that may recognize a protein containing that sequence. Peptides also have many biological functions on their own, interacting with receptors to stimulate changes in cellular function, or altering the interactions between cells, for example.

Chemists developed the means to synthesize peptides early in this century (*UNIT 18.1*). The ability to create a molecule that possessed biological activity by organic synthesis is certainly a milestone in our science. For many years, however, peptide synthesis was practiced only by highly skilled and dedicated individuals, and the lengthy time required to obtain even simple peptides limited their use as reagents for biological experiments. Difficulties in purification effectively eliminated the study of complex peptides of more than 5 to 10 amino acids.

Several developments have revolutionized this field, fortunately, and they have brought peptide science into the realm of standard biological techniques available to many scientists. First was the development of solid-phase methods by Bruce Merrifield and subsequent improvements in the reagents available for synthesis that are detailed in *UNIT 18.1*. Second was the design and construction of automated instruments to perform the repetitive steps of the solid-phase methodology, culminating in the 1980s with instruments that provided nearly global accessibility to synthetic peptides of ever increasing length.

Another advance was the optimization of reversed-phase HPLC as a separation method for the rapid purification and analysis of synthetic peptides (*UNIT 11.6*). A future unit in this chapter will focus on medium-to-large scale peptide purification by HPLC methods. In addition, recent advances in mass spectroscopy (Chapter 16) have provided another critical analytical tool to verify the products of synthesis.

A convenient, low-cost method for creating large numbers of related peptides or for assembly of a set of peptides that together span an entire protein to search for the epitope recognized by an antibody that binds to the full-length protein is provided by the synthesis of peptides on a multipin apparatus (*UNIT 18.2*).

Frequently, genomic studies lead to the discovery of an open reading frame (ORF), predicting a protein sequence that might have an activity of interest. One step that could follow would be the generation of an antibody to use as a reagent to identify the protein product of the gene in a sample isolated from the organism under study. A small peptide of 10 to 15 amino acids can frequently be used as an antigen to stimulate antiserum production. This requires picking the best sequence out of an ORF of hundreds of amino acids and preparing the conjugate for injection into an animal for generation of the antiserum (*UNIT 18.3*).

As the procedures employed in solid-phase peptide synthesis have improved, researchers' desire to attempt the preparation of longer sequences has grown. Methods to ligate

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medium- to large-sized peptides together through defined chemistry are presented in *UNIT 18.4*. Success in this approach has opened the door to a variety of new synthetic objectives.

The chemistry and strategy utilized to prepare peptide dendrimers (*UNIT 18.5*) for use as immunogens, designed protein mimics, new reagents for drug discovery, or new biomaterials has been an outgrowth of solid-phase peptide synthesis. Methods for both Boc and Fmoc syntheses are described here. In addition, several procedures for the formation of cyclic peptides are presented, including derivatization of Lys and Cys residues.

Units planned for future expansion of this chapter will cover synthesis of derivatives such as phosphopeptides or other modification that can be done at the resin-bound stage, the reactions necessary to form disulfide bridges at the appropriate points in peptides, the creation of derivatives in which selective peptide bonds have been replaced to create inhibitors or to stabilize against degradation in biological systems, methods for storage and handling of peptides, specialized methods for different types of resins for solid-phase synthesis, the creation of peptide libraries by chemical or biological approaches, the chemistry necessary to achieve the coupling of difficult or hindered peptides, and methods for the assembly of peptides of 100 amino acid residues in a single, long synthesis.

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COUPLING SYNTHETIC PEPTIDES TO A CARRIER PROTEIN USING A CARBODIIMIDE

ALTERNATE PROTOCOL 4

This procedure couples amino groups to carboxyl groups by way of activation of the carboxyl group with a water-soluble carbodiimide. Since the procedure is most easily performed in one step, peptides containing internal Asp, Glu, Lys, Tyr, or Cys residues should not be used. Also, in order to avoid making polymers of the peptide, the amino terminus should be blocked by acetylation during synthesis (see Basic Protocol 2). While this method has been used successfully, it is not considered to be the method of choice except in special situations.

Additional Materials (also see Basic Protocol 3)

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Pierce), used fresh or stored desiccated and frozen
0.1 N HCl

NOTE: Buffers containing amino or carboxyl groups should not be used in this procedure. According to some reports, buffers containing phosphate groups should also be avoided. Water is the safest choice as a solvent.

1. Dissolve 5 mg synthetic peptide in 1 ml water.
2. Add 25 mg EDC and carefully adjust pH to 4.0 to 5.0 by adding small amounts of 0.1 N HCl. Allow to react for 5 to 10 min at room temperature with gentle mixing.

   pH paper suffices to monitor this adjustment.
3. Dissolve 5 mg KLH in 0.5 ml water and add to solution from step 2. React 2 hr at room temperature with gentle mixing.
4. Dialyze against 4 liters of water overnight at 4°C. Use immediately.

COUPLING SYNTHETIC PEPTIDES TO A CARRIER PROTEIN PHOTOCHEMICALLY

ALTERNATE PROTOCOL 5

Most synthetic peptides today are made with 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry (see Fig. 18.1.1), and therefore the following protocol links the photoactive group to the free amino terminus of the peptide. An alternative approach if *t*-butyloxycarbonyl (Boc) chemistry is used is to link it to an ε-amino group of a terminal lysine (Gorka et al., 1989).

Additional Materials (see also Basic Protocol 3)

4-benzoyl benzoic acid (Sigma or Aldrich)
Quartz spectrophotometry cuvettes

1. Have the synthetic chemist who is making the peptide attach a benzoyl benzoic acid group to the amino terminus simply by treating the reagent as the terminal residue during normal synthesis.

   This blocks the amino terminus.
2. Dissolve 5 mg KLH in ~0.5 ml of 0.01 M sodium phosphate buffer, pH 7.5.
3. Add 2 mg of synthetic peptide containing the benzoyl benzoate adduct.
4. Place in a 1-cm quartz cuvette and irradiate with 366-nm light for 3 hr at a distance of 0.5 cm. Use immediately.

   The peptide can be used directly for immunization.

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REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Cysteine standard stock solution

Dissolve 26.3 mg cysteine hydrochloride monohydrate in 100 ml of 0.1 M sodium phosphate, pH 8.0 (*APPENDIX 2E*). Prepare immediately before use.

Ellman's reagent solution

Dissolve 4 mg Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid) (Pierce), in 1 ml of 0.1 M sodium phosphate, pH 8.0. Prepare immediately before use.

Glutaraldehyde solution, 0.15%

Add 30 µl of 25% aqueous glutaraldehyde solution to 5 ml of 50 mM sodium borate buffer, pH 8.0 (pH adjusted with HCl). Prepare fresh and use immediately. If the glutaraldehyde precipitates, check the pH. It should not be above 8.0; a slightly lower pH can be used (pH 7 to 8).

CAUTION: *Glutaraldehyde is a sensitizing agent that should be handled in a hood and only according to the recommendations in the Material Safety Data Sheet. When mixing solutions or performing reactions, keep the container covered to prevent vapors from escaping into the atmosphere.*

Guanidine·HCl, 6 M

Dissolve 1 g guanidine·HCl in 1 ml of 0.05 M sodium phosphate, pH 7.0. Store up to several weeks at room temperature.

The resulting 1.8-ml solution should be ~0.025 M phosphate/6 M guanidine·HCl at pH 7.0.

COMMENTARY

Background Information

Synthetic peptides are linear arrays of amino acids that in most instances possess a random structure in solution. While it is not difficult to produce antipeptide antibodies, it does not necessarily follow that the antibodies will recognize a protein containing the same stretch of sequence found in the peptide. In order for this to occur, the amino acids in the protein must be oriented to the antibody in a way similar to that of the synthetic peptide. This generally requires three basic features of the protein: (1) that the stretch of sequence be exposed to solvent; (2) that the sequence be a continuous stretch of amino acids; and (3) that it not possess a higher-order structure that renders it unrecognizable by the antibody population.

The large number of model protein structures now available indicate that almost all of the ionized groups in water-soluble proteins are on the protein surface. Asp, Glu, Lys, and Arg residues, on the average, comprise 27% of the protein surface and only ~4% of the protein interior. The fraction of residues at least 95% buried range from 0.36 to 0.60 for nonpolar residues and 0.01 to 0.23 for polar residues. Only 1% of Arg and 3% of Lys residues fall into the 95% buried range (Creighton, 1993). Therefore, it is reasonable to expect solvent-exposed areas of proteins to display relatively high levels of polar and charged residues, particularly Arg and Lys.

Proteins display three kinds of secondary structure: α-helices, β-sheets, and turns or loops (see *UNIT 17.1*). Turns or loops generally connect elements of α-helices and β-sheets, and can either fit one of several rather strict motifs with recognizable hydrogen bonding patterns or be of a more extended, random nature. These turn or loop structures appear to be most useful for antibody production because they tend to be found on the surface of proteins connecting larger arrays of helices and sheets, and they consist of continuous stretches of amino acids. Although many amino acid residues in helices and sheets are also exposed at the surface, the regular geometry of amino acids contained within them makes them less suitable for this purpose. For instance, in β-sheet structures the side chain of each successive amino acid in the β-sheet strand points in the opposite direction to the ones immediately preceding and following it. Thus, even if the amino acid side chains are not predominantly buried in the interior of

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Synthesis and Application of Peptide Dendrimers As Protein Mimetics

Peptide dendrimers are branched polymers with peptides attached centrally to a template or core matrix. They are synthesized as defined dendritic structures. Their molecular weights increase geometrically as a function of generation branching of monomers. Usually, 2 to 16 peptidyl branches of the same or different sequences are used to form a peptide dendrimer. Architecturally, peptide dendrimers belong to the multichained, cascade-shaped polymer family. Thus, they contain multiple N- or C-terminal ends that can be exploited as a platform for multitasks. Such a molecular architecture is different from conventional polypeptides or proteins that contain a single unbranched contiguous structure with two terminal ends. Peptide dendrimers are also different from organic star-burst dendrimers, which are spherically shaped and often consist of >16 hyperbranched small organic monomers. However, the fundamental dendrimeric design consisting of monomers radiated from a center core is similar, conceptually, to the organic dendrimers.

Peptide dendrimers come in different forms and sizes. Their forms are determined by the templates and peptide structures while their sizes are determined by the numbers and lengths of peptide monomers. Although their sizes vary over a wide range from 3 to >100 kDa, most peptide dendrimers reported to date are in the range of 6 to 20 kDa.

A driving force in developing peptide dendrimers is the effort to mimic forms and functions of proteins. Thus far, peptide dendrimers have found applications in four active areas: (1) immunological applications as immunogens and antigens; (2) de novo design of artificial proteins; (3) protein mimetics as agonists and antagonists in drug discovery; and (4) new biopolymers and biomaterials. However, this unit will focus on the use of peptide dendrimers as protein mimetics.

The use of peptides to mimic a portion of a protein structure is a challenging and powerful tool in the discovery of new drugs. In native proteins, discontinuous bioactive peptide surfaces are held together in a particular conformation by the structural rigidity of the protein. One approach to mimicking the structural surface on the protein is to bring the potential peptide sequences together by assembling the peptide chains on a template as a peptide dendrimer. Recently, small peptides have been demonstrated to adopt well defined conformations when they are attached on a template (Schneider and Kelly, 1995). In the past 15 years, templates suitable for protein engineering have been developed. These templates are flexible dendrimeric peptides (Tam, 1988; Wrighton et al., 1997) and cyclic peptides (Mutter and Vuilleumier, 1989), as well as more rigid organic molecules (Unson et al., 1984; Sasaki and Kaiser, 1989).

In 1988, the multiple antigenic peptide (MAP) system was introduced in the authors' laboratory (Tam, 1988; Tam and Lu, 1989) as a novel approach to preparing peptide immunogens. The MAP consists of an inner core matrix built up of a large layer of Lys residues and a surface of peptide chains attached to the core matrix (Fig. 18.5.1). Because of its dendrimeric structure, MAP can be very useful as a template for assembling potential peptide surfaces. Furthermore, the effect of MAP on secondary structures has also been studied (Shao and Tam, 1995). These study results showed that the peptide dendrimer has an increased ordered helical structure, while the peptide monomer and the tetravalent lysinyl core matrix do not have any ordered structure. The synthetic schemes for preparing MAPs suitable for protein mimetics are the same as those for generating MAPs as peptide immunogens. In addition to the applications in immunology, examples in the literature

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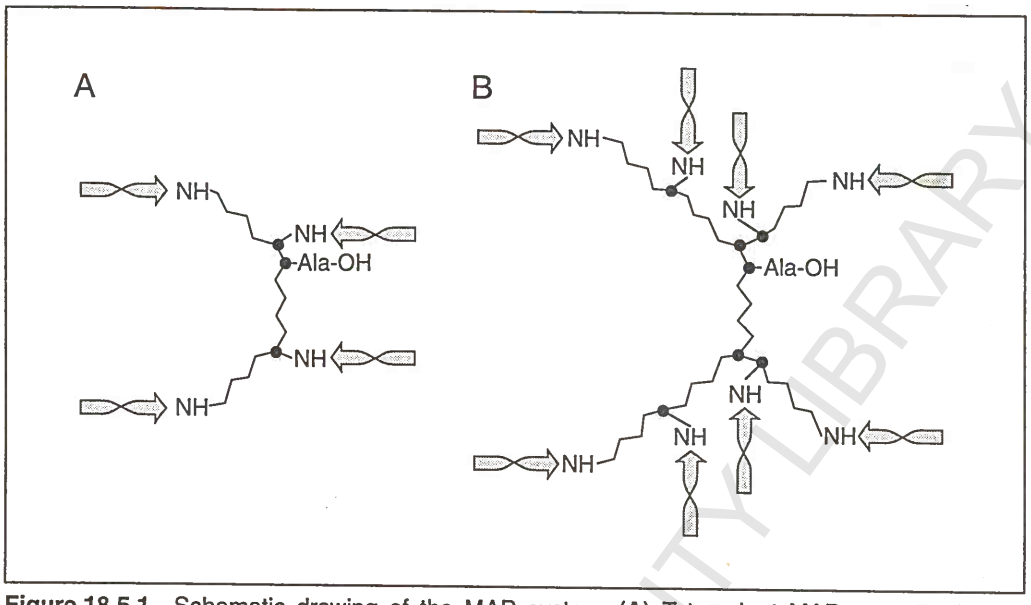

Figure 18.5.1 Schematic drawing of the MAP system. (**A**) Tetravalent MAP core matrix. (**B**) Octavalent MAP core matrix. The solid dots represent Lys and the ribbons with arrows represent the peptide.

have applied MAPs in areas such as inhibitors, artificial proteins, affinity purifications, and intracellular transport.

The MAP, which is chemically defined and homogeneous, was first intended as an approach to overcoming the limitations of the conventional method for producing anti-peptide antibodies. In the conventional approach, the peptide antigen is conjugated to a known large protein, or synthetic polymer carrier, to form a peptide-carrier conjugate. Although this approach has been used successfully in eliciting animal antibodies, it has several inherent limitations. First, only a small portion of peptide antigen is represented in the whole conjugate. Second, there is chemical ambiguity in the antigen composition and structure. Third, irrelevant epitopes and antibodies may be produced, and finally, carrier toxicity and carrier-induced epitope suppression may occur. The MAP systems have been used successfully to produce both polyclonal and monoclonal antibodies that specifically recognize native proteins. They have also been used to produce sera that have a significantly higher titer of antibodies than sera with antibodies against the same peptides conjugated to keyhole limpet hemocyanin (KLH) as a carrier protein (Tam, 1988).

Cyclic peptides are a powerful approach in the design of hormones and bioactive peptides, since favorable conformational constraints associated with biological activity can be fixed and conformational mobility diminished. In addition, the stability against biodegradation is increased. Cyclic peptides are also beneficial as peptide antigens, since they can elicit higher-quality antibodies than their linear counterparts. Based on these demands, the cyclic multiple antigen peptide (cMAP) approach (Spetzler and Tam, 1996; Zhang and Tam, 1997), which has branched multiple closed-chain architectures, was developed. The cMAP system is a superior approach for protein mimetics because the multiple constrained peptides can mimic bioactive conformations. Whether to select this approach over MAP depends on the properties of the peptides, but usually if the peptides are too small to adopt a stable conformation on their own, incorporation of a cyclic structure may be necessary.

Two methods for preparing MAP systems are described in this unit. The direct approach for preparing MAP systems is presented in the first two protocols, including the procedure

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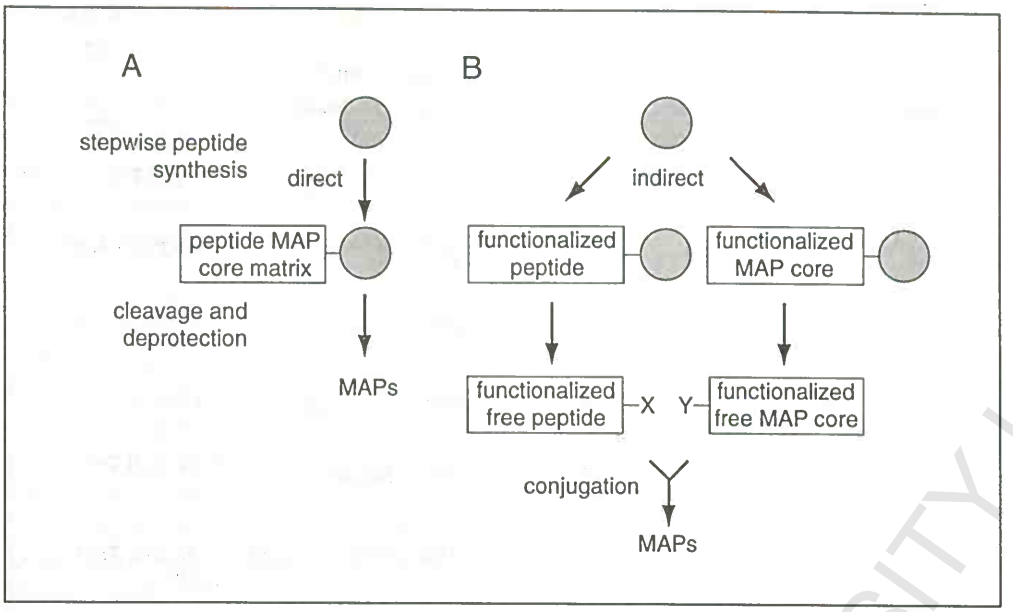

Figure 18.5.2 The direct and indirect approaches for preparing MAPs. Spheres represent the solid support; the MAP core matrix is Lys_2-Lys-Ala-OH or Lys_4-Lys_2-Ala-OH; X represents nucleophile; Y represents electrophile.

for *t*-butyloxycarbonyl (Boc) chemistry (see Basic Protocol 1) and the procedure for 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry (see Alternate Protocol). An indirect approach for preparing MAP systems, in which peptide and core matrix are synthesized separately and conjugated by several ligation methods, is then described (see Basic Protocols 2 and 3). The cMAP approach is also executed using either the direct or indirect approach, but requires an additional cyclization step to constrain the peptides after synthesis. The synthesis of cMAP is described (see Basic Protocols 4, 5, and 6), and the preparation of cyclic peptides is illustrated (see Support Protocols 4, 5, and 6). Support Protocol 1 describes the ninhydrin assay to assess the completeness of the coupling reaction. In most cases, MAP systems can be used directly after simple dialysis or desalting. Some immunological studies, however, require purified MAPs. Support Protocols 2 and 3 describe MAP system purification by dialysis and high-performance gel-filtration chromatography.

STRATEGIC PLANNING

Realizing the distinction between the direct and indirect approach is essential (Fig. 18.5.2) when synthesizing MAP systems. In the direct approach, the MAP core is formed by attaching several layers of lysine to the resin, which contains either Gly or Ala, using solid-phase peptide synthesis (Merrifield, 1963; *UNIT 18.1*). The peptide is then assembled onto this MAP core matrix (Basic Protocol 1 and Alternate Protocol). After the synthesis, the entire MAP is deprotected and cleaved from the resin. In the indirect approach, the MAP core and peptide are prepared and purified separately (Basic Protocols 2 to 3). Then, they are linked together in the absence of any side-chain protecting groups, after purification, to form a completed MAP. For preparing MAPs by either the direct or indirect approach, both the peptide synthesis and the ligation chemistry are easy to perform. Other features of the direct and indirect approach are discussed in the Commentary (see Background Information). However, the considerations for choosing a direct or indirect approach involve requirements for purity, properties of the peptide antigens, and time factors.

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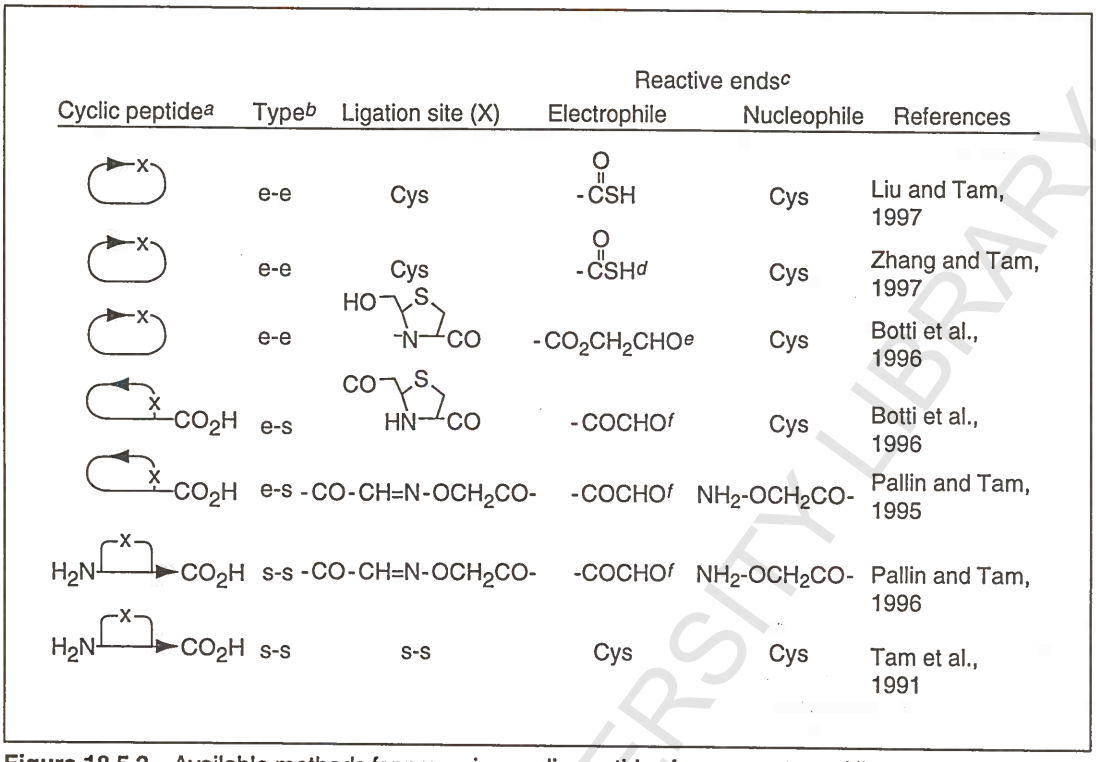

Cyclic peptide[a]	Type[b]	Ligation site (X)	Reactive ends[c] Electrophile	Nucleophile	References
	e-e	Cys	-CSH	Cys	Liu and Tam, 1997
	e-e	Cys	-CSH[d]	Cys	Zhang and Tam, 1997
	e-e		$-CO_2CH_2CHO$[e]	Cys	Botti et al., 1996
CO_2H	e-s		-COCHO[f]	Cys	Botti et al., 1996
CO_2H	e-s	$-CO-CH=N-OCH_2CO-$	-COCHO[f]	NH_2-OCH_2CO-	Pallin and Tam, 1995
H_2N CO_2H	s-s	$-CO-CH=N-OCH_2CO-$	-COCHO[f]	NH_2-OCH_2CO-	Pallin and Tam, 1996
H_2N CO_2H	s-s	s-s	Cys	Cys	Tam et al., 1991

Figure 18.5.3 Available methods for preparing cyclic peptides from unprotected linear peptides. Notes: (*a*) arrow indicates the direction of the amide bond; (*b*) types of cyclic peptides are designated as e-e, end-to-end; e-s, end-to-side chain; and s-s, side-chain-to-side-chain; (*c*) an electrophile/nucleophile pair is placed at the ends or side chain of the linear peptide (e.g., for formation of e-e cyclic peptide, the amino nucleophile is Cys and the carbonyl electrophile can be a thioester); (*d*) R = $(CH_2)_2$-$CONH_2$; (*e*) from periodate oxidation of a C-terminal glyceric ester; (*f*) from periodate oxidation of Ser and Thr attached to side chain of Lys.

For generating cMAPS, peptides on the core matrix need to be constrained as an additional step. A key tactic in the authors' strategy for preparing cMAP is the use of unprotected peptides as precursors. In the past few years, the authors' laboratory has developed a plethora of methods to form cyclic peptides from unprotected linear peptides in aqueous solution. Figure 18.5.3 lists those methods that are useful in preparing cMAPs. In planning of the cMAP synthesis, two questions need to be addressed: (1) how should the peptide antigen be constrained and (2) what are the suitable methods? The following are simple guidelines in the strategic planning for preparing cMAPs.

There are three general methods for constraining peptides in the cMAP system: end-to-end, end-to-side chain, and side chain-to-side chain (Figure 18.5.3). In one example (Basic Protocol 4) using the direct approach, cMAP is self-assembled via thiazolidine linkages after its unprotected precursor is synthesized by solid-phase synthesis. This procedure produces cyclic peptides that are linked in an end-to-side chain manner. A similar strategy can be applied for end-to-end or side chain-to-side chain methods by modifying the synthetic strategy accordingly. Peptides with a motif that favors a cyclic structure are highly recommended in this approach. Two other procedures (Basic Protocols 5 and 6) describe the use of an indirect approach for the synthesis of purified cMAP in two distinct steps. These protocols involve the synthesis of side chain-to-side chain and end-to-end cyclic peptides containing a free thiol, which are then linked to a MAP core matrix

A critical point in the preparation of both MAP and cMAP is the selection of the linear peptide sequences. Some of the available methods for identifying potential bioactive sequences include peptide library approaches (Tam et al., 1991; Houghten et al., 1991),

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simultaneous synthesis of a high number of peptides (*UNIT 18.2*), and computer-assisted modeling (*UNIT 18.3*).

The procedures described in this unit involve use of an automated peptide synthesizer (e.g., Perkin-Elmer Applied Biosystems). For Boc syntheses, a special hydrogen fluoride cleavage apparatus (Peptide Institute or Peninsula Laboratories) and hood are required. Manual procedures can also be used (Stewart and Young, 1984). A detailed discussion of peptide synthesizers and reagents is presented in *UNIT 18.1*.

CAUTION: Some chemicals used in these procedures are harmful. *N-N'*-dicyclohexylcarbodiimide (DCC) can cause severe allergy in sensitive persons. Avoid any skin contact with DCC! Benzotriazolyl *N*-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (BBTU) can be used as alternatives to DCC. Trifluoroacetic acid (TFA) is a toxic, volatile organic acid and should only be used under a hood. Besides being toxic, hydrogen fluoride (HF) is also highly corrosive to glass instruments. Never use glass instruments or containers in HF cleavage. Thick polyvinyl gloves and a protective face mask should be worn. More details about these chemicals can be found in the literature (Stewart and Young, 1984).

DIRECT Boc SYNTHESIS OF MAP SYSTEMS

BASIC PROTOCOL 1

In the direct approach for preparing MAP systems, the MAP core is first formed by attaching several levels of lysine onto an amino acid coupled to a resin matrix. The peptide sequence is then assembled stepwise on this MAP core resin. After peptide assembly is complete, the MAP system is cleaved from the resin support (Tam, 1988). The procedure for preparing MAP systems by the direct approach using Boc chemistry is presented in Figure 18.5.4 and Table 18.5.1. Solid-phase peptide synthesis (Merrifield, 1963) using

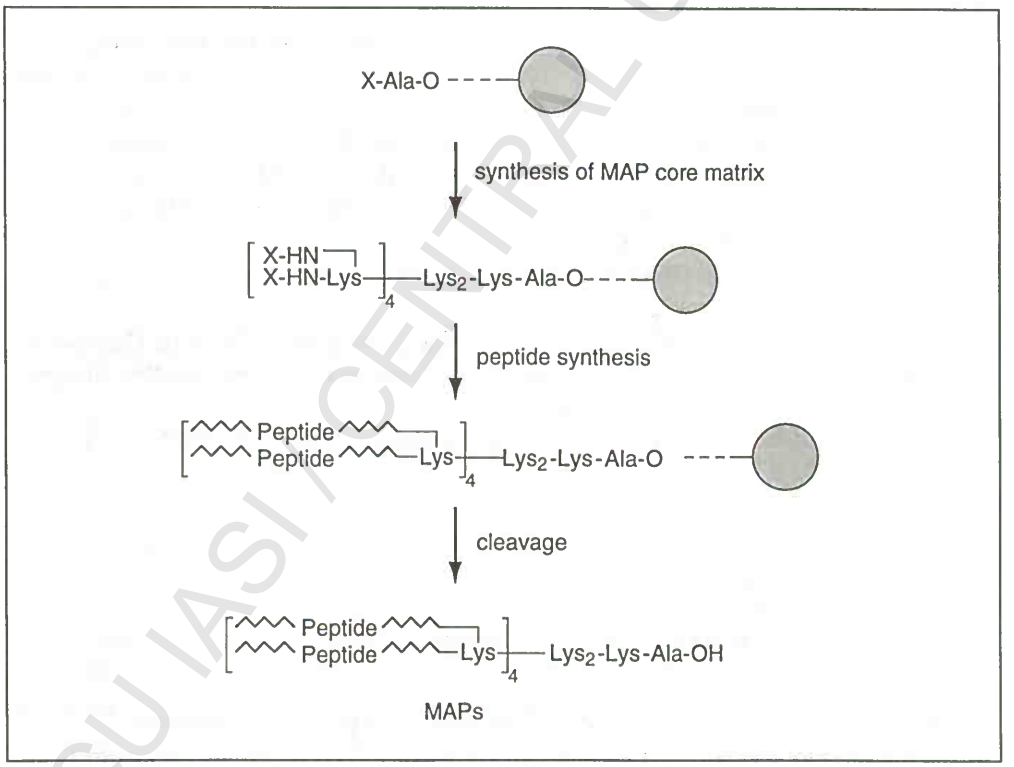

Figure 18.5.4 Direct approach of preparing MAPs with an octabranched core matrix. Sphere represents solid support; X represents Boc or Fmoc.

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Table 18.5.1 Schedule for Boc Solid-Phase Peptide Synthesis

Reaction	Description	Volume (ml/g resin)	Repetitions and time
1	DCM wash	15	3 × 1 min
2	50% (v/v) TFA/DCM prewash	15	1 × 1 min
3	50% (v/v) TFA/DCM deprotection	15	1 × 20 min
4	DCM wash (5×)	15	5 × 1 min
5	5% (v/v) DIEA/DCM prewash	15	1 × 1 min
6	5% (v/v) DIEA/DCM neutralization	15	1 × 5 min
7	DCM wash (5×)	15	5 × 1
8[a]	Boc-AA (3 eq) in DCM	5	1 × 1 min[b]
9	DCC (3 eq) in DCM	1	1 × 30 min[b]
10	Addition of DMF	15	1 × 30 min
11	DMF wash (2 times)	15	2 × 1 min
12	DCM wash (2 times)	15	2 × 1 min
13[c]	Ninhydrin test	—	5 min

[a]In case of coupling of Boc-Asn-OH, and Boc-Gln-OH, the DCC/HOBt coupling method should be used to avoid dehydration of unprotected side chain amide groups. Alternatively use Boc-Asn(Trt)-OH and Boc-Gln(Trt)-OH to minimize degradation side reactions. Boc-Arg(Tos)-OH should also be coupled with the DCC/HOBt method.

[b]Do not drain until the completion of reaction 10.

[c]If ninhydrin test does not show completion of coupling reaction (>99.5%), repeat reactions 7 to 13.

Boc chemistry involves prewashing the resin, removing the Boc protecting group, neutralizing the resin, coupling the next Boc–amino acid residue, and then repeating this cycle until the complete peptide sequence is assembled. Finally, the peptide is cleaved from the resin support.

Materials

Synthesis reagents (Table 18.5.1; prepared as described in Stewart and Young, 1984):
- Dichloromethane (methylene chloride, DCM)
- 50% (v/v) trifluoroacetic acid (TFA) in DCM (TFA/DCM)
- 5% (v/v) diisopropylethylamine (DIEA) in DCM (DIEA/DCM)
- 1.0 M *N,N′*-dicyclohexylcarbodiimide (DCC) in DCM (DCC/DCM)
- *N,N′*-dimethylformamide (DMF)

0.1 mmol/g Boc-β-Ala-OCH_2-PAM-resin (Bachem California)

Boc-Lys(Boc) (Bachem California)

Boc-amino acids (Boc-AA), with protected side-chain groups (Bachem California, Peninsula Laboratories) appropriate for synthesis of the desired peptide antigen, dissolved in dichloromethane (DCM).

10% (v/v) thiophenol in DMF *or* 10% (v/v) mercaptoethanol (2-ME)/5% (v/v) DIEA in DMF

p-cresol

p-thiocresol

Dimethyl sulfide (DMS)

Liquid hydrogen fluoride (HF), –78°C

99:1 (v/v) cold diethyl ether/2-mercaptoethanol (2-ME)

10% (v/v) acetic acid in water

Automated peptide synthesizer (e.g., Perkin-Elmer Applied Biosystems #ABI430A)

Manual reaction vessel (Pierce)

Hydrogen fluoride cleavage apparatus (Peptide Institute or Peninsula Laboratories)

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50-ml round-bottom flask
Coarse- and fine-porosity fritted glass funnels
50°C water bath

Prepare the core matrix

1. Place 0.5 g of 0.1 mmol/g Boc-β-Ala-OCH_2-PAM-resin in a reaction vessel in an automated peptide synthesizer. Begin the synthesis, using the synthesis reagents and directions outlined in Table 18.5.1, making the substitutions in reaction 8 of the table as described in step 2a, b, or c.

   Reactions 1 through 13 (Table 18.5.1) constitute one complete cycle; one cycle is required for each amino acid in the sequence. Some automated synthesizers may include the test to evaluate the coupling reaction in their cycle; if not, the ninhydrin test (reaction 13) will have to be performed manually (Support Protocol 1, Sarin et al., 1981).

   β-alanine is used as an internal standard for amino acid analysis which provides the molar ratios of other amino acids against β-alanine. Instead of β-alanine, other simple amino acids such as alanine can also be used.

2a. *To prepare a MAP core matrix with two lysine branches*: In reaction 8 of the first cycle, couple 0.15 mmol (52 mg) Boc-Lys(Boc) to the resin added in step 1. Proceed to step 3.

2b. *To prepare a MAP core matrix with four lysine branches*: Complete step 2a, then, in reaction 8 of the second cycle, couple 0.3 mmol (104 mg) Boc-Lys(Boc) to the resin from step 2a. Proceed to step 3.

   The substitution level doubles with each coupling of Boc-Lys(Boc).

2c. *To prepare a MAP core matrix with eight lysine branches*: Complete steps 2a and 2b, then, in reaction 8 of the third cycle, couple 0.6 mmol (208 mg) Boc-Lys(Boc) to the resin from step 2b. Proceed to step 3.

   Preformed MAP core matrix is also commercially available from Applied Biosystems, Bachem California, and Calbiochem-Novabiochem.

Synthesize the peptide on the MAP core matrix

3. Perform synthesis of the peptide on the MAP core matrix prepared in step 2a, b, or c by carrying out the reactions in Table 18.5.1 (one cycle of 13 reactions per amino acid), using, in reaction 8 of the table, 0.15 mmol of Boc-amino acids for a MAP core matrix with two branches, 0.3 mmol of Boc-amino acids for a MAP core matrix with four branches, or 0.6 mmol of Boc-amino acids for a MAP core matrix with eight branches, based on 0.05 mmol of starting Boc-β-Ala-OCH_2-PAM resin.

   Select the appropriate Boc-amino acids for the synthesis of the peptide antigen.

   For descriptive purposes, the peptide antigen IEDNEYTARQG (IG-11) is used in this protocol. For this peptide, the Boc-amino acids would be Boc-Gly, Boc-Gln, Boc-Arg (Tos), Boc-Ala, Boc-Thr(Bzl), Boc-Tyr(BrZ), Boc-Glu(OBzl), Boc-Asn, Boc-Asp (OBzl), and Boc-Ile for the synthesis of IG-11-MAP. The peptide chain would be assembled in sequence G, Q, R, A, T, Y, E, N, D, E, I. The most commonly used side-chain-protecting groups for Boc amino acids are: Ser and Thr, benzyl ether (Bzl); Asp and Glu, benzyl ester (OBzl); Asn, and Gln, unprotected or trityl (Trt) amide; Met, unprotected; Tyr, 2-bromobenzyloxycarbonyl (BrZ); Arg, toluenesulfonyl (Tos); Lys, 2-chlorobenzyloxycarbonyl (ClZ); His, N^{im}-2,4-dinitrophenyl (Dnp); Trp, N^{in}-formyl, Cys, p-methylbenzyl (MeBzl), or acetamidomethyl (Acm). Some special side-chain-protecting groups can also be found in the literature listed at the end of this unit (see Literature Cited).

4. After synthesis of the peptide is complete, transfer the resin to a manual reaction vessel and remove the Dnp from peptide residues with N^{im}-Dnp protecting groups using 10% thiophenol in DMF (three 8-hr treatments, 5 ml each), or 10% 2-ME/5% DIEA in DMF (two 30-min treatments, 5 ml each).

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5. Remove N-terminal Boc groups by treating for 20 min with 10 ml of 50% TFA/DCM, neutralize the resin with 10 ml of 5% DIEA/DCM, and then wash the resin five times, each time with 10 ml DCM (procedure similar to "reactions 1 to 4" in Table 18.5.1). Place the reaction vessel in a desiccator, and dry the resin under a vacuum.

   Boc groups would be removed in HF cleavage. It is advisable, however, to remove these t-butyl protecting groups prior to HF cleavage to avoid generating long-lived, reactive t-butyl cations during HF cleavage.

Cleave the peptides

6. Place 300 to 500 mg of MAP resin in the reaction vessel of the hydrogen fluoride cleavage apparatus. Melt *p*-cresol and *p*-thiocresol by placing bottles in 50°C water bath. Add 0.75 ml of the *p*-cresol and 0.25 ml of the *p*-thiocresol to the reaction vessel. Once this mixture has cooled to room temperature and solidified, add a magnetic stir-bar and 6.5 ml DMS to the reaction vessel and mix by stirring for 2 min at low speed without splattering resin on the reaction vessel.

7. *For low HF cleavage:* Add liquid HF at –78°C to give a final volume of 10 ml 7.5/2.5/65/25 (v/v/v/v) *p*-cresol/*p*-thiocresol/DMS/HF. Equilibrate the mixture to 0°C by immersing the reaction vessel in an ice bath and stirring. Stir the mixture vigorously for 2 hr at 0°C, and remove HF and DMS under a vacuum. Proceed to step 8 for high HF cleavage, or work up reaction as in step 9.

   CAUTION: *HF cleavage must be performed in a well ventilated chemical fume hood. Thick polyvinyl chloride gloves and a protective face mask must be used.*

   The outside wall of the vessel should have a predetermined mark for 10 ml.

8. *For high HF cleavage:* Recharge the mixture with 14 ml liquid HF at –78°C to give a final total volume of 15 ml HF/*p*-cresol/*p*-thiocresol. Stir the reaction mixture 1 hr at 0°C. Remove HF under a vacuum using a water aspirator at 0°C.

   See precautions for working with HF (step 7 annotation)

9. *Work up reaction:* Wash the residue by adding 30 ml of cold 99:1 diethyl ether/2-ME to the reaction vessel to remove *p*-thiocresol and *p*-cresol. Filter the resin and the precipitated peptide with a fritted-glass funnel in a 50-ml round-bottom flask. Discard the ether wash (the peptide will be distributed partially in the reaction vessel and partially in the fritted-glass funnel). Dissolve the crude peptide on both the reaction vessel and the fritted-glass funnel with 100 ml of 10% acetic acid, collect the filtrates, and lyophilize the solution to dryness. Store at –70°C.

ALTERNATE PROTOCOL

DIRECT Fmoc SOLID-PHASE SYNTHESIS OF MAP SYSTEMS

Fmoc synthetic chemistry is another widely used method for solid-phase peptide synthesis. The procedure for preparing MAP systems (Defoort et al., 1992) by the direct approach using Fmoc chemistry is presented in Figure 18.5.4 and Table 18.5.2. The basic procedure in Fmoc solid-phase peptide synthesis involves prewashing the resin, removing Fmoc protecting groups, and coupling the next Fmoc-amino acid residue. This cycle is repeated until peptide elongation is completed, and the peptide has been cleaved from the resin support.

Materials

Fmoc-Ala-Wang-resin (*p*-alkoxybenzyl alcohol resin preloaded with Fmoc-alanine at 0.3 to 0.5 mmol alanine/g resin; Bachem California)

Fmoc synthesis reagents

N,N'-dimethylformamide (DMF)

20% (v/v) piperidine in DMF (Pip/DMF)

0.5% (w/v) 1-hydroxybenzotriazole (HOBt) in DMF (HOBt/DMF)

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Table 18.5.2 Schedule for Fmoc Solid-Phase Peptide Synthesis

Reaction	Description	Volume (ml/g resin)	Repetitions and time
1	DMF wash	15	2 × 1 min
2	20% (v/v) Pip/DMF prewash	15	1 × 3 min
3	20% (v/v) Pip/DMF deprotection	15	1 × 20 min
4	DCM wash	15	6 × 1 min
5	0.5% HOBt/DMF wash	12	2 × 1 min
6[a]	Fmoc-AA (3 eq) in DMF	12	1 × 1 min[b]
7	HOBt (3 eq) in DMF	1	1 × 1 min[b]
8	DCC (3 eq) in DCM	1	1 × 60 min
9	DMF wash	15	5 × 1 min
10[c]	Ninhydrin test	—	5 min

[a]Coupling procedure for all amino acids.

[b]Do not drain until the completion of reaction 7.

[c]If ninhydrin test does not show completion of coupling reaction (>99.5%), repeat reactions 5 to 7.

Fmoc-Lys(Fmoc) (Bachem California; Peninsula Laboratories)
Acetic anhydride
Fmoc-amino acids (Fmoc-AA) with protected side-chain groups (Bachem California; Peninsula Laboratories) appropriate for synthesis of the desired peptide antigen, dissolved in DMT
Cleavage solution (see recipe), prepare fresh and prechill to 0°C
Anhydrous methyl *t*-butyl ether, cold
Anhydrous diethyl ether
10% (v/v) acetic acid in water
Automated peptide synthesizer (e.g., Perkin-Elmer Applied Biosystems #ABI430A)
Manual reaction vessel (Pierce)
Coarse- and fine-porosity fritted glass funnels

Prepare the resin

1. Place 0.5 g of 0.3 to 0.5 mmol/g Fmoc-Ala-Wang-resin in a reaction vessel in an automated peptide synthesizer. Couple 0.055 mmol (0.32 mg) Fmoc-Lys(Fmoc) onto the resin by carrying out reactions 1 to 9 in Table 18.5.2, adding the Fmoc-Lys(Fmoc) in reaction 6 of the table. Omit the ninhydrin test step.

   Reactions 1 through 10 (Table 18.5.2) constitute one complete cycle; one cycle is required for each amino acid in the sequence. Some automated synthesizers may include the test to evaluate the coupling reaction in their cycle; if not, the ninhydrin test will have to be performed manually (Support Protocol 1, Sarin et al., 1981).

   The commercially available Fmoc-Ala-Wang-resin usually has a loading of 0.3 to 0.5 mmol Fmoc-Ala/g resin. This content, however, is too high for synthesis of AMP (see Background Information). To prepare a resin with 0.1 mmol lysine/g resin at the first level in MAP core, only 0.055 mmol of Fmoc-Lys (Fmoc) should be used. This incomplete coupling will result in the loading of ~0.1 mmol lysine/g resin.

2. Add 15 ml DCM and 0.75 ml acetic anhydride to the reaction vessel and incubate 1 hr to terminate unreacted amines. Wash the resin five times with 10 ml DMF (1 min each) as in Table 18.5.2, reaction 9.

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As a result of the previous incomplete coupling step, an excess of free amino groups will be on the resin. This step will acylate (cap) free amino groups prior to coupling the next level of lysine.

Prepare the core matrix

3a. *To prepare a MAP core matrix with four or eight branches*: In reaction 6, couple 0.3 mmol (177 mg) Fmoc-Lys(Fmoc) to the resin from step 2 by following the reaction steps in Table 18.5.2. Proceed to step 3b (for core matrix with eight branches) or step 4 (for core matrix with four branches).

3b. *To prepare a MAP core matrix with eight branches*: In reaction 6, couple 0.6 mmol (354 mg) Fmoc-Lys(Fmoc) to the resin from step 3a by following the reaction steps in Table 18.5.2, adding the Fmoc-Lys(Fmoc) at reaction 6 in the table. Proceed to step 4.

Preformed MAP core matrix can be purchased from Applied Biosystems or Calbiochem-Novabiochem.

Synthesize the peptide on the MAP core matrix

4. Perform synthesis of the peptide on the MAP core matrix prepared in step 3a or b by carrying out the reactions in Table 18.5.2 (one cycle of 10 reactions per amino acid to be added) using, in reaction 6 of the table, 0.6 mmol of Fmoc-amino acids for a MAP core matrix with four branches or 1.2 mmol of Fmoc amino acids for a MAP core with eight branches.

The most commonly recommended side-chain-protecting groups for Fmoc-amino acids are: Ser, Thr, and Tyr, t-butyl ether (tBu); Asp and Glu, t-butyl ester (OtBu); Asn, Gln, trityl (Trt) amide; Arg, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (PMC) or 4-methoxy-2,3,6-trimethylhenzenesulfonyl (Mtr); Lys, tert-butyloxycarbonyl (Boc); His, N^{im}-trityl (TrT) Trp, and Met, unprotected; Cys, trityl (Trt) thioether or acetamidomethyl (Acm). All are commercially available.

5. After completing the synthesis, perform reactions 1 to 4 in Table 18.5.2, to remove the Fmoc group on the amino group of N-termini (last coupling) with 20% pip/DMF. Wash the resin with 10 ml DMF (three times, 1 min each) and 10 ml DCM (three times, 1 min each).

Cleave the peptides

6. Transfer peptide/resin mixture to a manual reaction vessel. Add prechilled cleavage solution to the peptide/resin at a ratio of 10 ml cleavage solution per 0.2 to 0.4 g peptide/resin. Stir mixture 1.5 to 2 hr at room temperature.

Other cleavage solutions can be used. Reagent K [82.5:5:5:2.5:5 (v/v/v/v/v) TFA/thioanisole/phenol/1,2-ethanedithiol (EDT)/H_2O] can be used for peptides that contain multiple Arg and Trp residues. For simple peptides, a cleavage solution of 5% H_2O/95% (v/v) TFA can be used.

7. Filter the peptide/resin mixture through a coarse-porosity fritted glass funnel, and wash the resin three times, each time with 5 ml TFA. Concentrate the filtrate to a small volume (~2 ml) under a vacuum.

8. Precipitate peptide with 30 to 50 ml cold anhydrous methyl *t*-butyl ether.

9. Filter the peptide precipitate through a fine-porosity fritted glass funnel, and wash the peptide three times, each time with 10 ml anhydrous diethyl ether. Dry the crude peptide (in the glass funnel) under a vacuum.

10. Dissolve the crude peptide in 10% acetic acid and lyophilize to dryness. Store at –70°C.

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INDIRECT Boc AND Fmoc SYNTHESIS OF MAP SYSTEMS USING THIOL CHEMISTRY

MAP systems have been successfully prepared by the direct approach, but they can also be synthesized by an indirect approach. In this method, the peptide antigen and MAP core matrix are prepared and purified separately and then linked together through functional groups on both elements to form a MAP system (Fig. 18.5.2 and Fig. 18.5.5; Lu et al., 1991). The indirect approach combines the advantages of solid-phase peptide synthesis with those of conjugation in a solution phase. An unprotected, purified peptide can be used in ligation. The orientation of peptides, particularly those derived from sequences at or near carboxyl termini, can be arranged as in native protein by linkage through the N-terminus to the core matrix. A variety of functional groups have been used for ligation to guarantee site-specific, chemically unambiguous MAP systems (also see Background Information). This protocol describes ligation through a sulfhydryl group of Cys in the peptide and a chloroacetyl group on the MAP core matrix. The same ligation chemistry is used to attach cyclic peptides to the MAP core matrix (see Basic Protocol 6).

Materials

Chloroacetic acid (Aldrich)
1.0 M *N,N'*-dicyclohexylcarbodiimide (DCC) in DCM (DCC/DCM)
Sulfhydryl reducing solution (see recipe)
Nitrogen source
1 M Tris·Cl
1 M NaOH
0.1 M NH_4HCO_3 or 10% acetic acid

Additional reagents and equipment for solid-phase peptide synthesis using Boc (see Basic Protocol 1) or Fmoc chemistry (see Alternate Protocol), purification

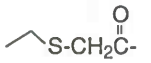
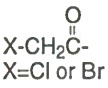
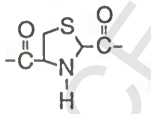
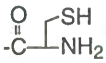
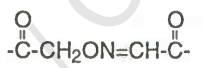
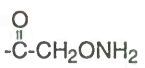
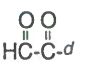
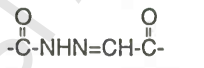
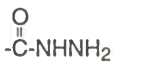
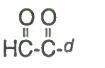
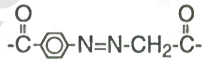
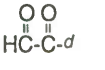
Peptide direction[a]	Ligation site	Reactive groups: Electrophile[b]	Reactive groups: Nucleophile[c]	References
C→N N→C				Lu et al., 1991
N→C				Shao and Tam, 1995
N→C				Shao and Tam, 1995
N→C				Shao and Tam, 1995
N→C				Spetzler and Tam, 1995

Figure 18.5.5 Ligation methods for preparing MAP or cMAP by the indirect approach. Notes: (*a*) The direction in which the peptide is attached to the core matrix; (*b*) the functional group is placed at the end of the peptide; (*c*) the functional group is placed at the end of the branched Lys residues; (*d*) Generated for $NaIO_4$ oxidation of Ser of Thr.

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of peptides via RP-HPLC (*UNIT 11.6*), dialysis (see Support Protocol 2), and high performance gel-filtration chromatography (see Support Protocol 3)

Prepare peptide antigen by solid-phase peptide synthesis

1. Synthesize the peptide using either Boc or Fmoc chemistry as in Basic Protocol 1 or the Alternate Protocol.

   For the ligation of peptide to a chloroacetylated MAP core matrix, an additional Cys should be attached to either C-termini (for peptide antigens derived from internal or N-terminal regions) or N-termini (for peptide antigens derived from the C-terminal region) of the peptide.

2. Purify the peptide using the methods in *UNIT 11.6* or in Support Protocols 2 and 3 of this unit.

Prepare chloroacetylated MAP core matrix

3. Prepare a MAP core matrix with four or eight lysine branches (see Basic Protocol 1, step 2), or Alternate Protocol, step 3).

4. Remove the N^{α}-terminal protecting group (see Basic Protocol 1, step 5, or Alternate Protocol, step 5).

5. Couple chloroacetic acid (4 molar equivalents) to the free amino groups of core matrix using 4 molar equivalents of DCC/DCM as a common amino acid coupling.

   This is done as in reactions 4 to 12 of Table 18.5.1 for Boc chemistry or reactions 4 to 9 of Table 18.5.2 for Fmoc chemistry, except that chloroacetic acid is being used instead of a protected amino acid.

6. Cleave the MAP core matrix from resin (see Basic Protocol 1, steps 6 to 9, for Boc-amino acids—using high HF cleavage—or Alternate Protocol, steps 6 to 8, for Fmoc-amino acids—using 95% (v/v) TFA in water as the cleavage solution).

7. Purify MAP core matrix using the methods in *UNIT 11.6* or in Support Protocols 2 and 3 in this unit.

Conjugate peptide antigen with chloroacetylated MAP core matrix

8. Dissolve 28 μmol peptide (from step 2) in 5 ml sulfhydryl reducing solution. Keep this solution under nitrogen and adjust pH to 6.0 to 6.5 with 1 M Tris·Cl. Flush the solution with nitrogen for 30 min, then seal the solution (no air contact) for 2 hr.

   Before the peptide is conjugated to the chloroacetylated MAP core matrix, the disulfide bond of the terminal Cys should be reduced to a sulfhydryl group by treatment with tributylphosphine (Bu_3P). Dithiothreitol (DTT) cannot be used for this purpose because DTT may react with the chloroacetylated core matrix.

   Note that this reaction is performed off-resin and in solution phase. On-resin conjugation can be performed by modifying the sequence of these reactions and the use of an aqueous-compatible resin support, e.g., PEG-modified resin, which is available commercially.

9. Add 4 μmol of the tetrabranched chloroacetylated MAP core matrix (from step 7) to the peptide solution (from step 8). Adjust the pH to 8.5 to 9.0 with 1 M NaOH. Stir the mixture 45 min at room temperature.

10. Dialyze the MAP system conjugate sequentially against 2 liters of 0.1 M NH_4HCO_3 or 10% acetic acid and then deionized water at room temperature, 8 hr each change. Lyophilize to dryness and store at –70°C.

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This dialysis procedure is different from that described in Support Protocol 2 because the peptides have already been purified.

11. Remove any byproducts by purifying the MAP conjugate by high-performance gel-filtration chromatography (Support Protocol 3).

INDIRECT Boc AND Fmoc SYNTHESIS OF MAP SYSTEMS USING CARBONYL CHEMISTRY

BASIC PROTOCOL 3

Another method for preparing the MAP system by the indirect approach is to exploit the selectivity of other weak bases, particularly in the condensation reaction with aldehydes (Fig. 18.5.5; Shao and Tam, 1995). Two types of weak bases can be used. The first type consists of conjugated amines whose basicities are lowered by neighboring electron-withdrawing groups such as hydroxylamine and substituted hydrazines. These react with aldehyde to form oxime from hydroxylamine and hydrazones from hydrazines. The second type contains the 1,2-disubstituted patterns such as 1,2-aminoethanethiol derivatives of N-terminal cysteine and 1,2-aminoethanol derivatives of N-terminal threonine. Proline-like rings such as thiazolidine or oxazolidine are formed from the reaction between an aldehyde and cysteine or threonine, respectively. An aldehyde moiety on a peptide or MAP core matrix can be obtained by $NaIO_4$ oxidation of N-terminal Ser, Thr, or Cys under neutral conditions to give an α-oxoacyl group. The additional weak base available for conjugation is attached to the N-termini of the peptide so the orientation of the peptide is C to N. However, the weak base-aldehyde chemistry has also been used to ligate constrained peptides to a MAP core matrix in the preparation of cMAP (see Basic Protocol 5).

Materials

Boc-aminooxyacetic acid (see recipe) *or* Boc-succinic acid hydrazide (see recipe)
Benzotriazolyl N-oxytrisdimethylaminophosphonium hexafluorophosphate (BOP) (Richelieu Biotechnologies)
Diisopropylethylamine (DIEA; Aldrich)
0.01 M sodium phosphate buffer, pH 7 (*APPENDIX 2E*)
Sodium periodate (Aldrich)
Ethylene glycol (Aldrich)
0.4 M sodium acetate buffer; mix equal volumes 0.4 M sodium acetate and 0.4 M acetic acid
Dimethyl sulfoxide (DMSO; Aldrich)
Dimethyl formamide (DMF)
0.1 M NaOH or glacial acetic acid
Argon source
C8 RP-HPLC column (see *UNIT 11.6*)

Additional reagents and equipment for solid-phase peptide synthesis using Boc chemistry (Basic Protocol 1) or Fmoc chemistry (Alternate Protocol), and purification of peptides by semipreparative RP-HPLC (*UNIT 11.6*)

Prepare the peptide by solid-phase peptide synthesis

1. Synthesize the peptide using Boc or Fmoc chemistry as in Basic Protocol 1 or the Alternate Protocol.

2. Remove the N-terminal protecting group (see Basic Protocol 1, step 5, or Alternate Protocol, step 5).

3. Couple (on-resin) the Cys residue (for thiazolidine ligation; 4 molar equivalents) to the free amino group of the peptide using 4 molar equivalents of DCC/DCM as a

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common amino acid coupling (see Table 18.5.1, steps 4 to 12, for Boc chemistry, or Table 18.5.2, steps 4 to 9, for Fmoc chemistry). Couple Boc-aminooxyacetic acid (for oxime ligation) or Boc-succinic acid hydrazide (for hydrazone ligation; 4 molar equivalents) to the amino terminus of the peptide using 4 molar equivalents of 1:1 (molar:molar) BOP/DIEA, dissolved in DMF, as coupling reagent in reaction 9 of Table 18.5.1 (in place of the DCC).

For thiazolidine ring formation, an additional Cys residue is attached to the N-terminus of the peptide. For coupling through an oxime or hydrazone bond, the weak base is also placed at the N-terminus of the peptide.

Prepare the glyoxyl-MAP core matrix

4. Prepare the tetravalent MAP core matrix (Lys_2-Lys-Ala) by the stepwise solid-phase synthesis method using either Boc chemistry (see Basic Protocol 1 and Table 18.5.1) or Fmoc chemistry (see Alternate Protocol and Table 18.5.2).

   Normal resin loading, ranging from 0.3 to 0.9 mmol/g, is recommended since only four coupling steps are required.

5. Remove the Boc group with 50% TFA/DCM (see Basic Protocol 1) or the Fmoc group with 20% pip/DMF (see Alternate Protocol).

6. Couple the fully protected Ser residue to the MAP core matrix using DCC and HOBt as described in step 3.

7. Cleave the Ser_4-MAP core matrix from the resin by the high HF procedure, HF/*p*-cresol (9:1, v/v), as described for Boc chemistry (see Basic Protocol 1, steps 6 to 9) or 95% (v/v) TFA in water as described for Fmoc chemistry (see Alternate Protocol, steps 6 to 8), and lyophilize the intermediate without purification.

8. Dissolve 5.86 µmol (5 mg) Ser_4-MAP core matrix (from step 7) in 2 ml 0.01 M sodium phosphate buffer, pH 7, and add 46.9 µmol (10 mg) sodium periodate in a 2-ml microcentrifuge tube.

9. Stir the mixture for 5 min at room temperature, then quench the reaction by adding 93.8 µmol ethylene glycol (5.3 µl).

10. Isolate the product by semipreparative RP-HPLC as described in *UNIT 11.6*.

Conjugate peptide with glyoxyl-MAP core matrix

11. Prepare a 5 mM peptide stock solution in water using the peptide from step 3 and a 5 mM glyoxy-MAP stock solution in water using the purified MAP from step 10.

12. Add 400 µl of 0.4 M sodium acetate buffer and 40 µl of the 5 mM glyoxy-MAP stock solution (0.2 µmol glyoxy-MAP) to 400 µl of the 5 mM peptide stock solution (2 µmol peptide). Then, add 800 µl DMSO in the case of oxime and hydrazone ligation and 800 µl DMF in the case of thiazolidine ligation.

13. Adjust pH of the solution to 5.7 (for oxime and hydrazone ligation) or 4.5 (for thiazolidine ligation) using 0.1 M NaOH or glacial acetic acid.

14. Stir the mixture under argon for 8 hr at room temperature, and monitor the completion of the ligation on a C8 RP-HPLC column (*UNIT 11.6*).

15. Purify the MAP conjugate by semipreparative HPLC (*UNIT 11.6*). Lyophilize to dryness and store at –70°C.

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NINHYDRIN TEST

SUPPORT PROTOCOL 1

The ninhydrin assay is used to check for completeness of the coupling reaction (Sarin et al., 1981). It is performed at the end of each synthesis cycle, usually as described here.

Materials

Ninhydrin test reagents: Solution A/B mixture and Solution C (see recipe)
60% (v/v) ethanol in H_2O
0.5 M tetraethylammonium chloride in dichloromethane (DCM)

10 × 75–mm test tubes
100°C heating block
Pasteur pipet containing glass wool plug

1. At the end of each synthesis cycle (see Table 18.5.1 and Table 18.5.2), remove ~10 mg wet resin from the reaction vessel. Dry the resin 1 hr under vacuum in a desiccator.
2. Weigh a 3- to 5-mg sample of dry resin into a 10 × 75–mm test tube. Add 100 µl of the solution A/B mixture and 25 µl solution C. To another tube add only the ninhydrin test reagents without resin. Mix.
3. Heat both tubes 10 min in a heating block at 100°C. Cool tubes in cold water.
4. Add 1 ml of 60% ethanol to the tubes. Mix thoroughly and filter through a Pasteur pipet containing a glass wool plug.
5. Rinse the resin twice with 0.20 ml of 0.5 M tetraethylammonium chloride in DCM. Combine the filtrates.
6. Dilute the combined filtrate to 2 ml with 60% ethanol.
7. Measure the A_{570} of the sample filtrate against the reagent blank.

For routine monitoring, an effective extinction coefficient of 1.5×10^4 may be used.

PURIFICATION OF MAP SYSTEM BY DIALYSIS

SUPPORT PROTOCOL 2

The chemistry of solid-phase peptide synthesis has been significantly improved since it was first introduced in 1963. Now synthesis of peptides of 100 or more residues with satisfactory yield and purity is possible. In general, crude synthetic MAP systems (after the cleavage step) are pure enough for many purposes (e.g., for production of antisera). When highly purified MAP systems are required (e.g., for vaccines for humans), the crude synthetic product must be purified to near homogeneity. MAP systems can be purified by dialysis, gel-filtration chromatography, RP-HPLC (*UNIT 11.6*), and high-performance gel-filtration chromatography (Support Protocol 3). This protocol details the special procedure required for the sequential dialysis of MAP systems.

Materials

Crude MAP system (see Basic Protocol 1, 2, or 3, or Alternate Protocol)
0.1 M NH_4HCO_3/$(NH_4)_2CO_3$ (pH 8.0) in 8 M and 2 M urea
1 M acetic acid
Dialysis tubing (e.g., Spectra/Por 6, MWCO 1000, Spectrum)

1. Dissolve crude MAP system in 100 ml of 0.1 M NH_4HCO_3/$(NH_4)_2CO_3$ (pH 8.0) in 8 M urea.
2. Load the peptide solution into dialysis tubing.

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3. Dialyze the peptide solution sequentially against 2 liters of each of the following solutions, for 8 hr each, at room temperature:

   0.1 M $NH_4HCO_3/(NH_4)_2CO_3$ (pH 8.0) in 8 M urea
   0.1 M $NH_4HCO_3/(NH_4)_2CO_3$ (pH 8.0) in 2 M urea
   H_2O
   1 M acetic acid.

4. Remove peptide solution from dialysis tubing. Lyophilize to dryness. Store at –70°C.

SUPPORT PROTOCOL 3

PURIFICATION OF MAP USING HIGH-PERFORMANCE GEL-FILTRATION CHROMATOGRAPHY

In this protocol, a crude MAP system is dissolved in potassium phosphate and purified over a specialized gel-filtration column by HPLC.

Materials

10 mg crude MAP system (Basic Protocol 1, 2, or 3, or Alternate Protocol)
0.1 M potassium phosphate, pH 7.0 (*APPENDIX 2E*)

Column for high-performance gel-filtration chromatography (prepacked Bio-Sil TSK 250, 300-mm length × 7.5-mm i.d.; mol. wt. range for separation: 10 to 300 kDa; Bio-Rad #125-0062)
Guard column (75-mm length × 7.5-mm i.d.; Bio-Rad #125-0061)

Additional reagents and equipment for HPLC (*UNIT 11.6*)

1. Dissolve 10 mg crude MAP system in 1 ml of 0.1 M potassium phosphate, pH 7.0.
2. Set up HPLC system with the Bio-Sil TSK column linked to the guard column. Set UV detector at 225 nm. Equilibrate the gel-filtration column with 0.1 M potassium phosphate, pH 7.0, at a flow rate of 0.5 ml/min.
3. Inject 200 µl of the crude MAP solution (from step 1). Elute peptide at a flow rate of 0.5 ml/min. Collect the purified MAP product.
4. Repeat injection (step 3) four times to purify all of the material. Pool the purified MAP product.
5. Lyophilize MAP fraction to dryness. Store MAP at –70°C.

BASIC PROTOCOL 4

DIRECT SYNTHESIS OF END-TO-SIDE CHAIN cMAP

This approach is suitable for preparing cMAP with multiple end-to-side chain cyclic peptides. It combines solid-phase (on-resin synthesis) with solution-phase synthesis (off-resin cyclization; Spetzler and Tam, 1996). First, stepwise solid phase synthesis is used to assemble the peptides linked to the MAP core matrix on resin. The cMAP precursor is then cleaved from the resin, and its protecting groups removed. The peptide antigens are cyclized in cMAP in aqueous solution. The chosen sequences were peptide antigens derived from the V3 loop of the surface protein gp120 of human immunodeficiency virus-1 (HIV-1), MN strain. Two peptides with the sequence GPGRAFYTTKNIIG and KRKRIHIGPGRAFYTTKNIIG were used. The essential motif GPGR was placed in the middle of the antigen sequence. The cMAP precursor contains a tripartite design (Fig. 18.5.6). The first two parts are constant regions. Part 1 at the C-terminus contains a short peptide which can be used for identification, delivery to give an MHC class I molecule or a T-helper epitope, followed by a tetralysyl core matrix. Part 2 contains a specific chemical cleavage site AspPro that gives a monomeric cyclic peptide to verify the yield of the intrachain cyclization. This part may not be necessary if verification is

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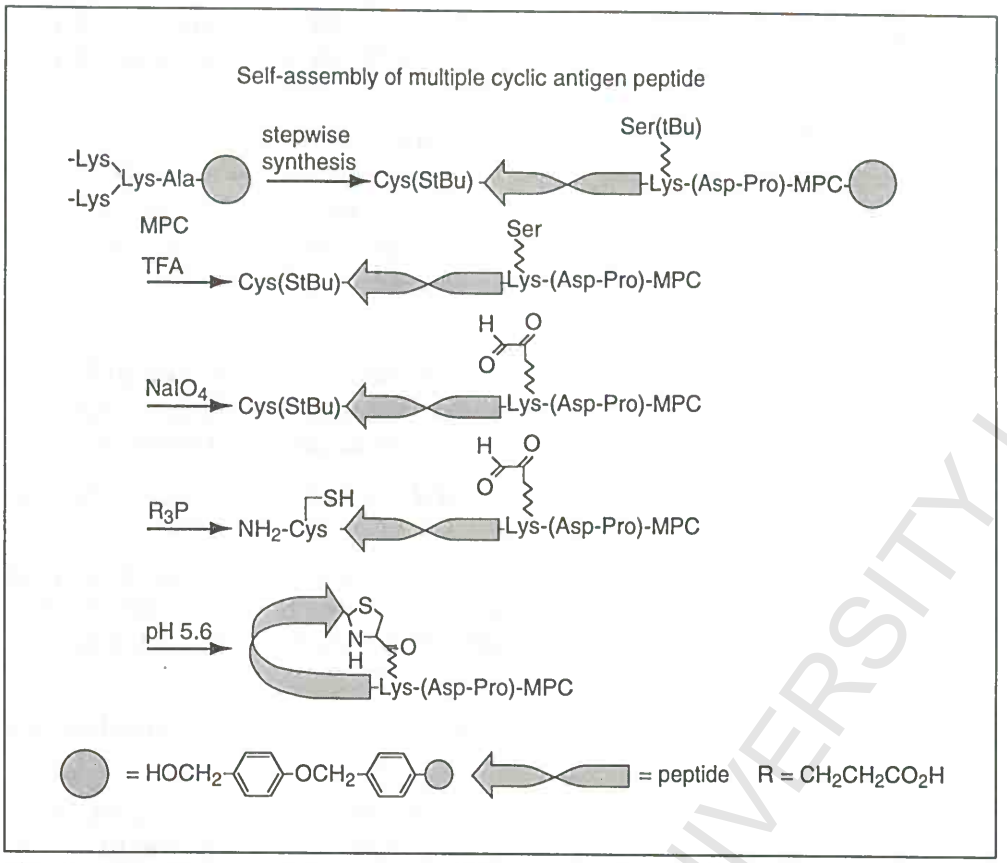

Figure 18.5.6 The scheme for preparing cMAP using the direct method.

not needed. The third part is the variable region and contains a peptide framed by a lysine at the COOH-terminus (ε-Lys) and a Cys at the amino terminus to give the end-to-side chain cyclic structure. The two mutually reactive functions are the α-Cys and a masked aldehyde attached to the side chain of the C-terminal lysine. Conversion to the aldehyde under mild aqueous conditions will permit an end-to-side chain cyclization via thiazolidine (Fig. 18.5.6). The protecting strategy for the two reactive moieties, Cys and aldehyde, is designed to be compatible with Fmoc chemistry (Alternate Protocol and Table 18.5.2) and the removal of these moieties under aqueous conditions. Sulfenyl tertbutyl is chosen for Cys as Cys(StBu) because it can be removed under aqueous conditions by trialkylphosphine and is stable to oxidation by periodate. The masked form of the aldehyde is the 1,2-aminoalcohol of a Ser residue anchored on the lysyl side chain. It can be converted to an aldehyde by periodate oxidation. The use of trialkylphosphine in the scheme has two advantages. First, it inhibits intermolecular disulfide formation as a side reaction, and second, it allows a one-pot reaction for unmasking the thiol protecting group and cyclization.

Materials

Fmoc-Lys[methyltrityl(Mtt)] (Calbiochem-Novabiochem)
Fmoc-Lys(Fmoc) (Bachem California)
Fmoc-Cys(StBu) (Calbiochem-Novabiochem)
1% (v/v) TFA/5% (v/v) triisopropylsilane (TIS; Aldrich) in DCM
Cleavage solution: 92.5/2.5/2.5/2.5 (v/v) trifluoroacetic acid (TFA)/triisopropyl silane (TIS) (Aldrich)/thioanisole (Aldrich)/H_2O, prepared fresh
0.01 M sodium phosphate buffer, pH 6.8 (*APPENDIX 2E*)

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Sodium periodate (Aldrich)
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Calbiochem-Novabiochem)
10 mM sodium phosphate, pH 6.8 (*APPENDIX 2E*)
10 mM sodium acetate buffer, pH 4.2 (*APPENDIX 2E*)
70% (v/v) formic acid in H_2O

Additional reagents and equipment for peptide synthesis (Alternate Protocol and Table 18.5.2), semipreparative HPLC (*UNIT 11.6*), and assay of free sulfhydryls with Ellman's reagent (*UNIT 18.3*)

Perform on-resin synthesis of the cMAP precursor

1. Synthesize the cMAP precursor starting from Fmoc-Ala-Wang resin (0.5 g, 0.1 mmol/g) manually or via an automated peptide synthesizer using the synthesis reagents and directions outlined in Table 18.5.2 and described in Alternate Protocol.

   The order of assembly of the precursor is: the MAP core matrix, the cleavage site Asp-Pro, Fmoc-Lys(Mtt), peptide sequence, and Fmoc-Cys(S-tBu). See Figure 18.5.6.

2. Remove (on-resin) the side-chain-protecting 4-methyltrityl (Mtt) group of Lys using 1% TFA/5% TIS in DCM using the procedure described in step 6 of the Alternate Protocol. Discard the TFA/TIS/DCM solution and neutralize the TFA-amine salt on-resin by performing reactions 4 to 7 of Table 18.5.1.

3. Couple Fmoc-Ser(tBu) to the lysyl side chain by DCC and HOBt procedure as a common amino acid coupling as in reactions 6 to 9 of Table 18.5.2.

4. Deprotect and cleave the precursor of cMAP from the resin by 92.5/2.5/2.5/2.5 (v/v) TFA/TIS/thioanisole/H_2O as outlined in the Alternate Protocol, steps 5 to 10. Lyophilize the peptide.

   Avoid thiol scavengers due to the presence of Cys(S-tBu).

Perform off-resin cyclization to form cMAP

5. Dissolve 0.44 µmol cMAP precursor (from step 4) in 2 ml 0.01 M sodium phosphate buffer, pH 6.8, and add 3.52 µmol (0.75 mg) sodium periodate in 27 µl water.

6. Stir the solution for 2 min, then purify the peptide by semipreparative HPLC as described in *UNIT 11.6*. Lyophilize the oxidized cMAP precursor.

7. Add 17.6 µmol (1.5 mg) TCEP in 47 µl of 10 mM sodium acetate buffer, pH 4.2, to the solution of oxidized cMAP in 1.5 ml of 10 mM sodium acetate buffer, pH 4.2.

8. Stir the mixture at 22°C for 48 hr, and monitor the progress of the cyclization by assay of free sulfhydryls with Ellman's reagent (*UNIT 18.3*) until all the thiols are consumed.

9. Purify cMAP by semipreparative HPLC as described in *UNIT 11.6*.

10. For determining the intrachain yield, add 0.5 ml 70% formic acid to 0.05 µmol of the lyophilized cMAP in a glass test tube. Incubate the solution either at 37°C for 48 hr or 60°C for 24 hr. Add 0.5 ml water and lyophilize the sample.

    This procedure will cleave the cyclic peptide at the AspPro site as a monomer.

11. Separate the monomeric sample by RP-HPLC (*UNIT 11.6*).

    The intrachain yield is calculated from the amino acid hydrolysis of the cMAP and the cyclic monomer.

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INDIRECT Fmoc SYNTHESIS OF SIDE CHAIN-TO-SIDE CHAIN cMAP USING CARBONYL CHEMISTRY

In this approach, a side chain-to-side chain cyclized peptide is attached to the core matrix through a lysyl side chain (Pallin and Tam, 1996). The sequence used is a peptide antigen chosen from the neutralizing determinant of the V3 loop of gpl20. The target amino acid sequence is IGPGP. The precursors, which contain three Lys residues, are introduced as Fmoc-Lys(Mtt) and Boc-Lys(Fmoc), respectively, for attaching three functional groups (hydroxylamine, Ser, and Cys(StBu) through the side chains (Fig. 18.5.7). An *O*-alkyl-hydroxylamine group is attached to the side chain of the first Lys before the peptide sequence. The second Lys is introduced after the peptide sequence, and a Ser moiety is coupled to the side chain as a masked aldehyde, which is oxidized to an aldehyde by $NaIO_4$. A cyclic peptide is formed by using the intramolecular oxime formation from the reaction between *O*-alkylhydroxylamine and the aldehyde. A Cys(StBu) residue on the

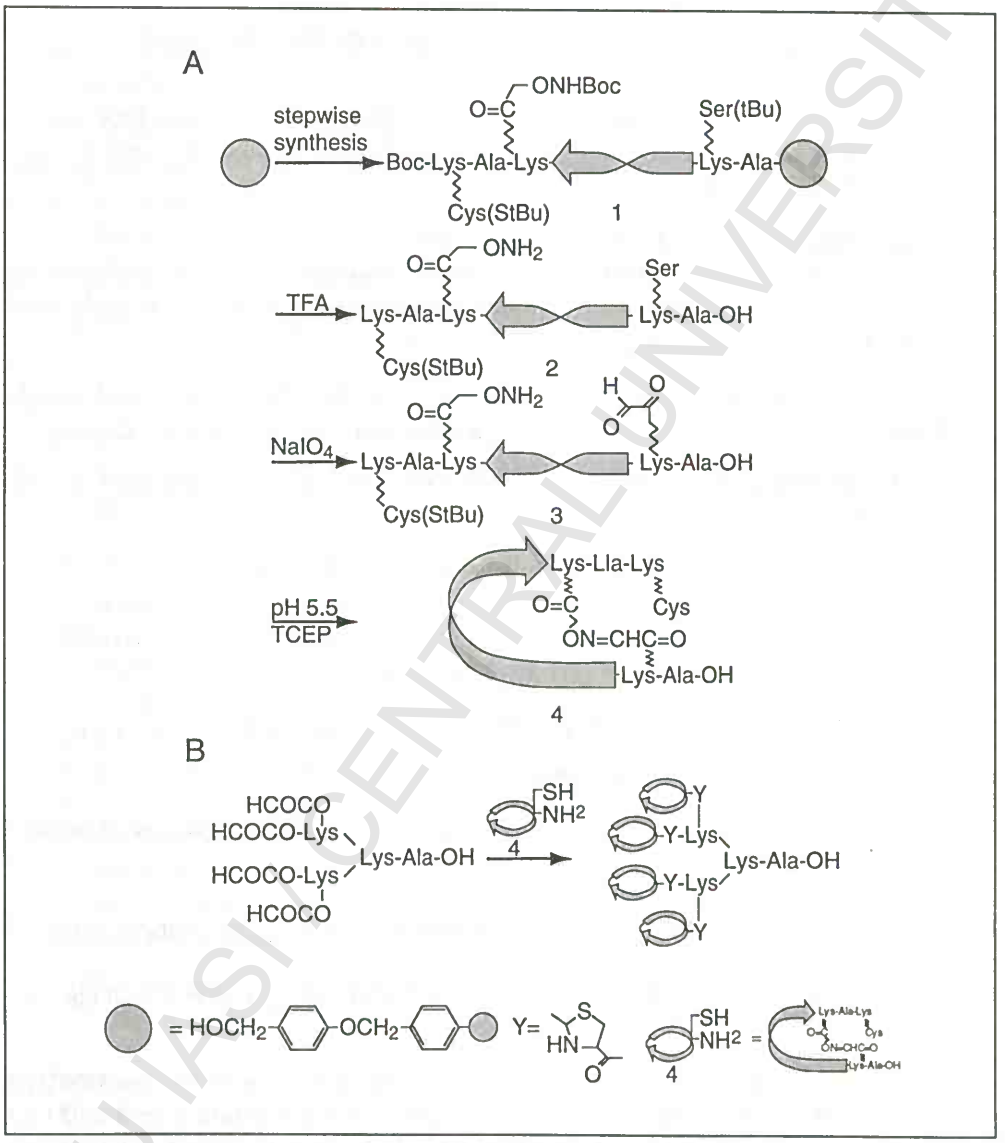

Figure 18.5.7 The scheme for preparing cMAP utilizing carbonyl chemistry and using the indirect method. (A) Oxime approach for preparing unprotected cyclic peptide antigen. (B) Attachment to MAP via thiazolidine linkage.

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side chain of the third Lys moiety is used to ligate the cyclic peptides to the aldehyde moieties of the MAP core.

Materials

Fmoc-Lys[methyltrityl(Mtt)] (Calbiochem-Novabiochem)
1% (v/v) TFA/5% (v/v) triisopropylsilane (TIS; Aldrich) in DCM
20% piperidine in DMF (pip/DMF)
Boc-Ser(tBu; Bachem California)
Boc-(aminooxy)acetic acid (Bachem California)
Boc-Lys(Fmoc; Bachem California)
0.01 M sodium acetate buffer, pH 7 (*APPENDIX 2E*)
Sodium periodate ($NaIO_4$;Aldrich)
Ethylene glycol
0.1 M NaOH
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Calbiochem-Novabiochem)
0.05 M sodium acetate buffer, pH 6 (*APPENDIX 2E*)
Tetravalent MAP core containing aldehyde groups (see Basic Protocol 3)
Acetic acid

Additional reagents and equipment for peptide synthesis (see Alternate Protocol and Table 18.5.2), semipreparative and preparative HPLC (*UNIT 11.6*), and mass spectrometry (Chapter 16),

Perform on-resin synthesis of the cyclic precursor

1. Synthesize the linear unprotected peptide by the stepwise solid phase strategy using the Fmoc chemistry protocol (see Alternate Protocol and Table 18.5.2) and couple the first Lys residue as Fmoc-Lys(Mtt).
2. Remove the Mtt protecting group using 1% TFA/5% TIS in DCM and couple Boc-Ser(tBu) to the side chain of Lys as described in step 2 of Basic Protocol 4.
3. Synthesize the peptide sequence using the Fmoc strategy as described in the Alternate Protocol.
4. Incorporate the second Lys residue as the Fmoc-Lys(Mtt) derivative.
5. Couple Boc-(aminooxy)acetic acid to its side chain.
6. Couple the third Lys as a Boc-Lys(Fmoc) derivative.
7. Remove the Fmoc group by 20% pip/DMF (see Alternate Protocol).
8. Couple Fmoc-Cys(StBu) to the side chain of lysine.
9. Cleave and purify the peptide precursor as described for Fmoc chemistry (Alternate Protocol).

Perform off-resin cyclization to form side chain-to-side chain cyclic peptides via an oxime linkage

10. Dissolve the peptide in 0.01 M sodium acetate buffer, pH 7, to give a final concentration of 0.8 M.
11. Oxidize the 1,2-amino alcohol moiety to the glyoxyl derivative by addition of a 2 M excess of $NaIO_4$ for 2 min at room temperature. Quench reaction with a >20-fold excess of ethylene glycol.
12. Purify the peptide by semipreparative HPLC (*UNIT 11.6*) to remove the formaldehyde byproduct.

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13. Collect the product from semipreparative HPLC and adjust pH to 5.5 with 0.1 M NaOH, to effect the oxime cyclization.

14. Monitor the progress of the intramolecular cyclization by RP-HPLC (*UNIT 11.6*).

    The reaction is usually completed within 12 hr.

15. Characterize the cyclic peptide by mass spectrometry (Chapter 16).

Attach cyclic peptides to the MAP core matrix via a thiazoline linkage

16. Add 1.5 µmol (0.13 mg) TCEP to a solution of 0.3 µmol cyclic peptide in 0.5 ml of 0.05 M sodium acetate buffer, pH 6. Stir the mixture for 1 hr at room temperature.

17. Add 0.036 µmol tetravalent MAP core containing aldehyde groups (Basic Protocol 3) to the above mixture and adjust pH to 5 with acetic acid.

18. Stir the solution for 36 hr at 18°C, and purify the product by preparative HPLC as described in *UNIT 11.6*.

INDIRECT PREPARATION OF END-TO-END cMAP USING THIOL CHEMISTRY

BASIC PROTOCOL 6

In this protocol, the cyclic peptide is attached through its side chain to the core matrix (Zhang and Tam, 1997). The selected sequences were YGGFL and AVEIQFMHNLGK. First, the precursor of the unprotected cyclic peptide and the functionalized core matrix are synthesized by SPPS (Fig. 18.5.8). The cyclic peptide is generated by an intramolecular transthioesterification reaction in which the cyclization occurs under equilibration of ring chain tautomerization in aqueous solution. Sequentially, the end-to-end cyclic peptide is formed via an S- to N-acyl transfer reaction, and a Cys residue is generated. In this method, racemization is minimized, and the cyclization can occur in high concentration. The generated free thiol group of the Cys residue is then available for ligation. Preparation of the (chloroacetyl)lysyl MAP core matrix is described in Basic Protocol 2. In the second step, the thioether bond is formed between the cyclic peptide and the core matrix at slightly basic pH.

Materials

3-thiopropionic acid (Aldrich)
MBHA (*p*-methyl benzhydrylamine) or Gly-PAM resin (Calbiochem-Novabiochem)
1 mM dithiothreitol (DTT; Aldrich) in distilled water
20 mM Na_2HPO_4/10mM citric acid buffer, pH 7.5
Tris(2-carboxyethyl)phosphine, hydrochloride (TCEP; Calbiochem-Novabiochem)
0.2 M sodium phosphate buffer, pH 7.4 (*APPENDIX 2E*)/10 M ethylenediaminetetraacetic acid (EDTA; Aldrich)
Argon source
Tetravalent chloroacetyl(lysinyl) core matrix (see Basic Protocol 2)
Dimethylformamide (DMF)

Additional reagents and equipment for peptide synthesis (see Basic Protocol 1), preparative RP-HPLC (*UNIT 11.6*), and mass spectrometry (Chapter 16)

Perform on-resin synthesis of the cyclic precursor

1. Couple 3-thiopropionic acid to 0.5 g MBHA or Gly-PAM resin at 1.1 mmol/g by DCC/DCM in a reaction vessel (see Basic Protocol 1).

   Boc-Gly-PAM resin must be deprotected to give a free α-amine. The idea of this step is to form a reversible linker containing a thioester at the C-terminus after HF cleavage. For

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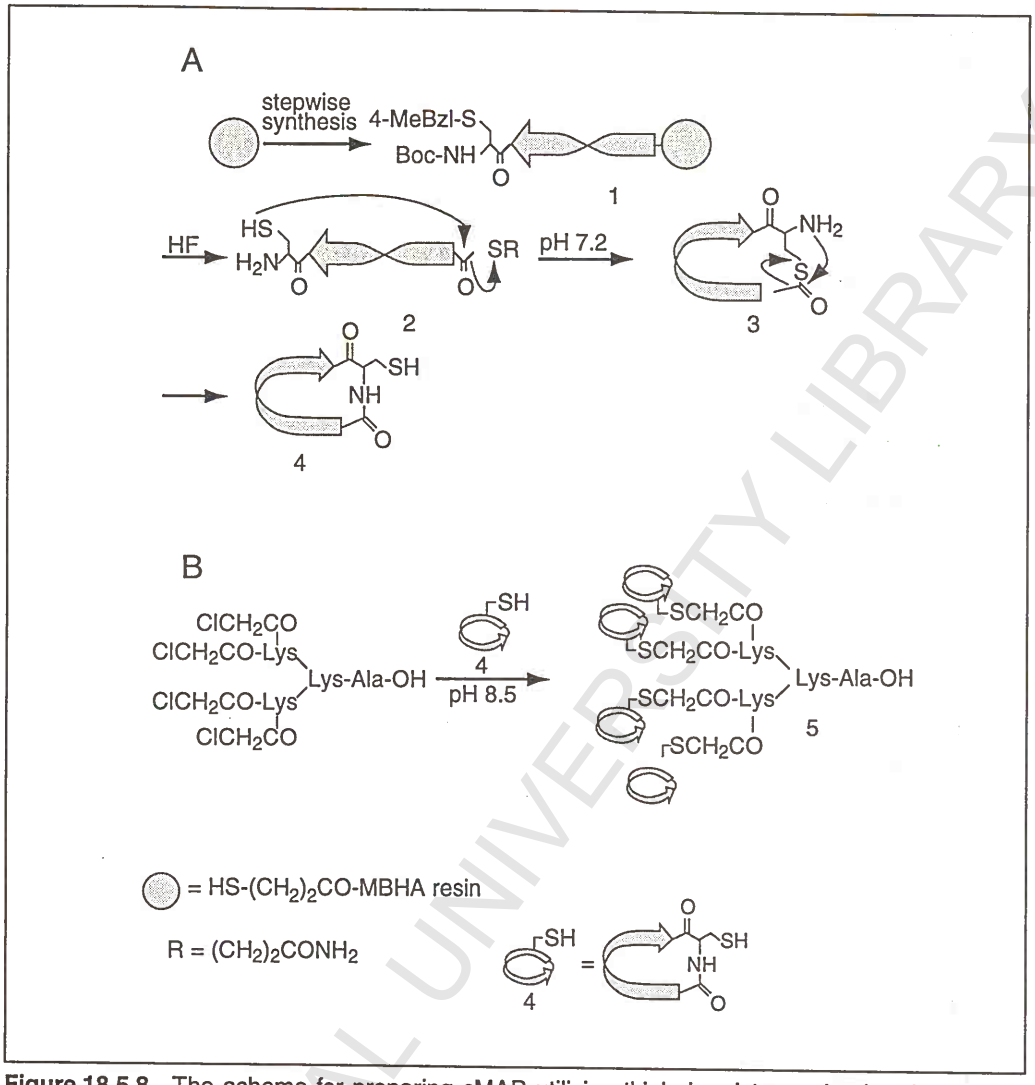

Figure 18.5.8 The scheme for preparing cMAP utilizing thiol chemistry and using the indirect method. **(A)** Thioester approach for preparing unprotected cyclic peptide antigen. **(B)** Attachment to MAP via thioalkylation.

an alternative method to prepare this resin, see recipe for thioester resin (HS-CH_2CH_2CO-MBHA-resin 1) for C-terminal thioester.

2. Add 1 mM DTT to the resin for 1 hr.

   Disulfide bonds may have been formed during the acylation reaction.

3. Synthesize the peptide sequence by stepwise solid phase synthesis (also see Fig. 18.5.7) either with an automated peptide synthesizer or manually using the synthesis reagents and directions outlined in Table 18.5.1 and Basic Protocol 1.

   Avoid the use of base-mediated coupling reagents such as benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOB), 2-(IH-benzotriazol-1-yl)-1, 1, 3,3-tetramethyluronium tetrafluorohorate (TBTU), and 2-(IH-benzotriazol-1-yl)-1,1, 3,3-tetramethyluronium hexafluorophosphate (HBTU), since the thioester is not stable to base.

4. Cleave the peptide thioester by the HF protocol as described in Basic Protocol 1. Purify the product by preparative RP-HPLC (*UNIT 11.6*). Lyophilize and characterize the product by mass spectrometry (Chapter 16).

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If the His residue is present in the sequence, use Boc-His(Tos) instead of Boc-His(DNP) which requires thiolysis to release the DNP protecting group.

Perform off-resin cyclization to form an end-to-end cyclic peptide containing an X-Cys ligation site

5. Dissolve 3.5 µmol linear peptide thioester (from step 4) in a solution of 2 ml of 20 mM Na_2HPO_4/10mM citric acid buffer, pH 7.5, containing 3.5 µmol (1.04 mg) TCEP.
6. Stir the solution for 6 hr, and monitor the progress of the intramolecular cyclization by RP-HPLC (*UNIT 11.6*).
7. Purify the cyclic peptide by preparative RP-HPLC as described in *UNIT 11.6*. Characterize and lyophilize the product.

Attach cyclic peptides to the MAP core via a thioether linkage

8. Dissolve 28 µmol cyclic peptide (from step 7) in 1.25 ml of 0.2 M sodium phosphate buffer containing 10 µM EDTA, pH 7.4.
9. Purge the solution with argon for 10 min.
10. Add 1.4 µmol (1.1 mg) tetravalent (chloroacetyl)lysinyl core matrix (prepared as described in Basic Protocol 2) predissolved in 0.75 ml DMF.
11. Stir the mixture for 24 hr at room temperature, then purify the cMAP by preparative RP-HPLC as described in *UNIT 11.6*.
12. Characterize cMAP by mass spectrometry (Chapter 16).

PREPARING END-TO-END CYCLIC PEPTIDES VIA AN X-CYS LIGATION SITE

SUPPORT PROTOCOL 4

In this protocol, an unprotected cyclic peptide containing an X-Cys ligation site is prepared (Liu and Tam, 1997). The sequence is a peptide antigen derived from the V3 loop of the gp120 of IIIB. The peptide precursor is synthesized on a thioester benzhydryl resin which, after HF cleavage, generates C^{α}-thiocarboxylic acid. A Cys residue activated as Cys(Npys) is incorporated at the amino-terminus. First, the end-to-end peptide is cyclized from formation of a mixed acyl disulfide which undergoes an intramolecular S,N-acyl transfer reaction. The generated hydrodisulfide (S-SH) can be reduced by thiolysis to give the native Cys-residue.

Materials

Thiobenzhydryl resin for C-terminal thioacid (see recipe)
Boc-Cys(Npys) (Bachem California)
25% acetonitrile/0.05% TFA in H_2O, pH 2
Sodium acetate, solid
Dithiothreitol (DTT; Aldrich)

Additional reagents and equipment for peptide synthesis (see Basic Protocol 1 and Table 18.5.1) and RP-HPLC (*UNIT 11.6*)

1. Perform on-resin synthesis of the cyclic precursor containing an X-Cys ligation site. Synthesize the precursor of the cyclic peptide on a thiobenzhydryl resin using the Boc-chemistry protocol as described in Basic Protocol 1 and outlined in Table 18.5.1. Cap the amino-terminal with Boc-Cys(Npys), and cleave and deprotect the peptide by HF.

The resulting precursor is a peptide with a Cys(Npys) residue at the N-terminal and a thiocarboxylic acid at the C-terminal. The other side chains are unprotected.

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2. Initiate the cyclization after HF cleavage (see Basic Protocol 1) in 25% acetonitrile in H_2O containing 0.05% TFA, pH 2, giving a final concentration of 1.75 μM with respect to the peptide solution. Monitor the progress of the cyclization by RP-HPLC (*UNIT 11.6*).

   During the cyclization, a sulfur-sulfur exchange occurs first and a yellow color appears due to the release of Npys-H.

3. Adjust the solution to pH 6 by using solid sodium acetate to complete the cyclization reaction.

4. After 10 min, add 1 eq DTT to the solution to reduce the cysteinyl hydrodisulfide to cysteine, and monitor the reaction by RP-HPLC (*UNIT 11.6*).

5. Purify the cyclic peptide by preparative RP-HPLC as described in *UNIT 11.6*.

SUPPORT PROTOCOL 5

PREPARING END-TO-END CYCLIC PEPTIDES VIA AN X-THIAPROLINE LIGATION SITE

In this example, the unprotected peptide precursor is synthesized on an Fmoc-amino acid-*O*-CH_2-cyclic acetal resin (Botti et al., 1996). The peptide sequence is as follows: GRAFVTIGKIG derived from the V3 loop of gpl20, HIV-1. During the TFA cleavage, a glyceric ester is generated at the C-terminal. The diol of the glyceric ester is converted to glycoaldehyde by oxidation with $NaIO_4$. The precursor contains a Cys(StBu) residue at the N-terminus while all the other side chains are unprotected. Two steps are involved to form thiaproline. First, the thiazolidine ring is generated from the reaction between an aldehyde moiety and the 1,2-aminothiol of the N-terminal Cys. Then, an *O*- to *N*-acyl transfer reaction occurs through a tricyclic ring contraction to give a peptide bond. The thiazolidine ring formation is mediated after $NaIO_4$ oxidation and removal of the StBu protecting group, and the intramolecular *O*- to *N*-acyl transfer reaction occurs upon readjusting the pH to 5.9.

Materials

Fmoc-Gly-*O*-CH_2-cyclic-acetal resin (see recipe for acetal resin)
Cleavage solution: 91:3:3:3 (v/v) TFA/H_2O/thioanisole/anisole
$NaIO_4$
0.01 M sodium phosphate buffer, pH 5.5, 5.7 and 6 (*APPENDIX 2E*)
Tributyl phosphine (Bu_3P)
Isopropyl alcohol

Additional reagents and equipment for peptide synthesis (see Alternate Protocol 2 and Table 18.5.2) and RP-HPLC (*UNIT 11.6*)

1. Synthesize end-to-end cyclic peptides via a thiaproline ligation site. Assemble the peptide sequence on Fmoc-Gly-*O*-CH_2-cyclic-acetal resin by Fmoc chemistry using stepwise phase synthesis as described in the Alternate Protocol and Table 18.5.2. Cap the amino-terminal with Boc-Cys(StBu).

2. Cleave and deprotect the precursor as in the Alternate Protocol, steps 6 to 10, using a cleavage solution containing 91:3:3:3 TFA/H_2O/thioanisole/anisole and allowing the reaction procedure to proceed for 2.5 hr. Lyophilize the product (Alternate Protocol, step 10).

3. Add 30 μmol $NaIO_4$ in 0.3 ml 0.01 M sodium phosphate buffer, pH 5.7, to 7 μmol peptide glyceric ester also dissolved in 1 ml 0.01 M sodium phosphate buffer, pH 5.7. Stir the solution for 30 min, and purify the product by semipreparative RP-HPLC (*UNIT 11.6*).

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4. Dissolve 1.18 μmol peptide-glycoaldehyde (from step 3) in 0.2 ml 0.01 M sodium phosphate buffer, pH 5.5, and add 10.9 μmol tributyl phosphine (Bu_3P) in 30 μl isopropyl alcohol (1:10 v/v dilution of Bu_3P in isopropyl alcohol). Stir and monitor the progress of the cyclization by analytical RP-HPLC (*UNIT 11.6*). Purify and lyophilize the product.

   The starting product forms two products corresponding to the cis and trans isomers of the cyclic peptide.

5. Redissolve the purified peptide in 0.2 ml 0.01 M sodium phosphate buffer, pH 6, and monitor the amide formation by analytical RP-HPLC (the *O*- to *N*-acyl transfer can take up to several days). If the reaction is slow, warm up the solution to 40°C. Purify the product and analyze by HPLC.

PREPARING END-TO-SIDE CHAIN CYCLIC PEPTIDES VIA A THIAZOLIDINE OR OXIME LINKAGE

SUPPORT PROTOCOL 6

In these two examples (Pallin and Tam, 1995; Botti et al., 1996), the precursor contains a Lys residue which is introduced as an Fmoc-Lys(Mtt) for attaching Ser through its side chain. The sequences are peptide antigens ranging from 5 to 26 amino acids in length and are derived from the V3 loop of gp120, HIV-1. The 1,2-amino alcohol function of Ser acts as a masked aldehyde and can be converted to glyoxyaldehyde after treatment with $NaIO_4$. A Cys(StBu) moiety is incorporated at the N-terminus to generate a thiazolidine ring while Boc-(aminooxy)acetic acid is incorporated to form an oxime bond. All the other side chains are unprotected. The thiazolidine ring is formed by first oxidizing the amino-terminal Ser to an aldehyde moiety and then removing the StBu protection group by TCEP. In generating cyclic oxime peptides, cyclization occurs after oxidation to the aldehyde.

Materials

Fmoc-Lys(Mtt)
1% (v/v) TFA/5% (v/v) triisopropylsilane (TIS; Aldrich) in DCM
Boc-Ser(tBu)
Cleavage solution: 92.5/2.5/2.5/2.5 (v/v) TFA/TIS/thioanisol/H_2O
Sodium periodate
0.01 M sodium phosphate buffer, pH 6.8 (*APPENDIX 2E*)
0.01 M sodium acetate buffer, pH 4.2 (*APPENDIX 2E*)
Tris(2-carboxyethyl)phosphine, hydrochloride (TCEP; Calbiochem-Novabiochem)

Additional reagents and equipment for peptide synthesis (see Alternate Protocol and Table 18.5.2), RP-HPLC (*UNIT 11.6*), and mass spectrometry (Chapter 16)

1. Synthesize end-to-side chain cyclic peptides via a thiazolidine or oxime linkage. Assemble the peptide segment on a Wang resin by the Fmoc-chemistry protocol using stepwise peptide synthesis as described in the Alternate Protocol and outlined in Table 18.5.2.

   The order is as follows: X-protected peptide sequence-Lys[Boc-Ser(tBu)]βAla, where X = Boc-Cys(StBu) or Boc-NH-O-CH_2-CO-.

2. Couple the Lys residue as Fmoc-Lys(Mtt) and remove the Mtt protection group with 1% TFA,/5% TIS in DCM. Couple Boc-Ser(tBu) to the side chain of Lys (Alternate Protocol).

3. Cleave and deprotect the peptide (see Alternate Protocol, steps 6 to 10) using 92.5/2.5/2.5/2.5 TFA/TIS/thioanisol/H_2O and allowing the reaction to proceed for 1 hr. Lyophilize the crude product (Alternate Protocol, step 10).

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4. Add 1.55 μmol sodium periodate (0.33 mg) in 3.5 μl H_2O to 0.76 μmol peptide precursor dissolved in 1 ml 0.01 M sodium phosphate buffer, pH 6.8. Shake the solution for 2 min, and purify the product by semipreparative RP-HPLC (*UNIT 11.6*).

   For oxime formation, the intramolecular cyclization occurs after oxidation to the aldehyde. Purify and characterize the product.

5. Dissolve 0.94 μmol peptide in 1 ml 0.01 M sodium acetate buffer, pH 4.2, and add 9.3 μmol TCEP (2.3 mg) dissolved in 23 μl 0.01 M sodium acetate buffer, pH 4.2. Stir the solution, and monitor the cyclization by analytical RP-HPLC (*UNIT 11.6*). After purification by semipreparative RP-HPLC (*UNIT 11.6*), characterize the product by mass spectrometry (Chapter 16).

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see ***APPENDIX 2E****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acetal resin

Add 0.2 g sodium bicarbonate to a suspension of chloromethylated polystyrene (1% divinylbenzene) copolymer (Merrifield resin, purchased, e.g., from Bachem California; 0.76 mmol/g, 0.5 g) in 3 ml DMSO. After stirring for 7 hr at 155°C, wash the resin with water, methanol, DMF, DCM, and diethyl ether and then dry under vacuum. To confirm the conversion of the chloromethyl resin to the aldehyde resin, use FT-IR analysis, which should show the typical carbonyl absorption at 1700.5 cm^{-1}, and microanalysis, which should show that the resin does not contain any chlorine. The benzaldehyde resin (0.59 mmol/g, 1.0 g) is suspended in 10 ml anhydrous dimethoxyethane with 1 g glycerol and 10 mg *p*-toluenesulfonic acid. After 24 hr at 90°C, wash the resin with a 1:1 (v/v) mixture of 3% sodium bicarbonate and 1,4-dioxane, followed by water, dioxane, DMF, methanol, DCM, and diethyl ether. Dry under vacuum. Use FT-IR analysis to monitor the disappearance of the carbonyl absorbance at 1700.5 cm^{-1}. To functionalize the acetal resin with a Fmoc-amino acid, activate the Fmoc-amino acid, e.g., Fmoc-Gly-OH (5.58 mmol, 1.66 g) using DCC (3 mmol, 0.62 g), dimethylaminopyridine (0.28 mmol, 35 mg), and HOBt (0.28 mmol, 44 mg) for 20 min in 10 ml DMF. Insoluble dicyclohexylurea is then removed, and the solution is added to a suspension of the glycerol-acetal resin (1.0 g) in 5 ml DMF. After 18 hr, wash the resin three times each with dioxane, DMF, methanol, DCM, diethyl ether and then dry under vacuum.

Microanalysis will indicate 0.71% of nitrogen, corresponding to a substitution of 0.5 mmol/g. UV absorbance of the cleaved Fmoc (wavelength 301 nm, I = 7840) will indicate a substitution of 0.5 mmol/g.

Boc-aminooxyacetic acid

Add 10 mmol carboxymethylamine hemihydrochloride (Aldrich) to a mixture of 20 ml dioxane, 10 ml water, and 10 ml 1 N NaOH on ice. Add 11 mmol di-*t*-butyl pyrocarbonate (Aldrich) to the above solution at 0°C and stir the solution overnight at room temperature. Adjust pH to 4 using diluted $KHSO_4$ and extract the product twice, each time for 30 min, with ethyl acetate. Dry the organic phase over $MgSO_4$. Evaporate the organic phase under vacuum, and recrystallize Boc-(aminooxy) acetic acid (Boc-$NHNH_2$) from ethyl acetate–hexane.

Boc-succinic acid hydrazide

On ice, dissolve 42 mmol Boc-$NHNH_2$ (Aldrich) in 20 ml dichloromethane (DCM) and add 40 mmol succinic anhydride. Then add 10 mmol diisopropylethylamine (DIEA) to the above solution at 0°C, and stir the solution for 6 hr at room

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temperature. Remove DCM under vacuum, and dissolve the residue in 60 ml ethyl acetate. Wash the solution twice with 15 ml 5% $KHSO_4$, twice with 10 ml saturated NaCl solution, and finally with 10 ml water. Dry the organic phase over $MgSO_4$, and evaporate the organic phase under vacuum. Wash the oily residue three times, each time with 20 ml ether-hexane and dry under vacuum.

Cleavage solution for Fmoc protecting group

90 parts trifluoroacetic acid (TFA)
6 parts thioanisole
3 parts 1,2-ethanedithiol (EDT)
1 part anisole
Prepare fresh and chill to 0°C

The above reagents are available from Aldrich

Ninhydrin test reagents

Solution A: Dissolve 40 g reagent grade phenol in 10 ml absolute ethanol.

Solution B: Dissolve 65 mg potassium cyanide in 100 ml water. Dilute 2 ml of this solution to 100 ml with freshly distilled pyridine. Mix solution A and solution B, and store indefinitely at 4°C.

Solution C: Dissolve 2.5 g ninhydrin in 50 ml absolute ethanol. Store solution C indefinitely, in the dark.

Pyridine from any commercial source may be used to prepare solution B and must be distilled before use. Reflux 500 ml pyridine with 2 g ninhydrin for 2 hr, then distill the pyridine and store several months at 4°C, in a brown bottle (away from light).

Sulfhydryl reducing solution

4.25 ml 6 M guanidine·HCl
0. 25 ml 5% (w/v) EDTA (50 mg EDTA/ml)
0.5 ml 2% (w/v) tributylphosphine (Bu_3P) in propanol
Prepare just before use

CAUTION: *Tributylphosphine (Bu_3P) is a highly toxic, volatile reducing agent and should only be handled in a chemical hood.*

Thiobenzhydryl resin 2 for C-terminal thioacid

Add thiolacetic acid (CH_3COSH, 30 mmol; Aldrich Chemical) and triethylamine (30 mmol; Aldrich Chemical) to a suspension of 4-methylbenzhydryl chloride resin (6 g, 3 mmol) in 60 ml DMF. React the mixture at 60°C for 12 to 18 hr until a negative silver chloride test (see below) of the resin indicates that no free chloride groups are present. Wash the resin three times each with DMF, DCM, CH_3OH, DCM, and DMF. Treat resulting resin with a mixture of cysteine methyl ester hydrochloride (9 mmol), triphenylphosphine (9 mmol), and DIEA (9 mmol) in DMF/DCM (3:1, v/v, 60 ml). After 2 hr, wash the resin thoroughly three times each with DMF, DCM, and CH3OH and dry under vacuum.

The yield from this procedure should be 6.3 g.

For the silver chloride test, remove ~20 mg of resin to a small glass test tube, add 0.5 ml of pyridine, and heat at 100°C in a heating block for 15 min in a well-ventilated hood. Cool and dilute with an equal volume of 0.1 N nitric acid. Add a few drops of 0.1 N silver nitrate solution. The presence of silver chloride precipitate indicates an incomplete reaction.

Thioester resin (HS-CH_2CH_2CO-MBHA-resin 1) for C-terminal thioester

Add 3-thiopropionic acid (1.2 ml, 13 mmol), HOBt (1.8 g, 13 mmol), and DIC (2 ml, 13 mmol) sequentially to a suspension of 4-methylbenzhydrylamine (MBHA) resin (6 g, 3.2 mmol) in 60 ml DMF. Shake the mixture at room temperature for 30 min until a ninhydrin test (see Support Protocol 1) of the resin indicates that no free

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amino groups are present. Wash the resin three times each with DMF, DCM, CH_3OH, DCM, and DMF. Treat resulting resin with a mixture of cysteine methyl ester hydrochloride (0.55 g, 3.2 mmol), triphenylphosphine (0.85 g, 3.2 mmol), and DIEA (0.68 ml, 3.9 mmol) in DMF/DCM (3:1, V/V, 60 ml). After 2 hr, wash the resin thoroughly three times each with DMF, DCM, and CH_3OH and dry under vacuum.

The yield from this procedure should be 6.3 g.

COMMENTARY

Background Information

The multiple antigen peptide (MAP) system was originally designed to replace the protein carrier and to overcome the limitations of conventional conjugation approaches (Tam, 1988, 1996; Posnett et al., 1988). The MAP system consists of three structural elements. A simple amino acid (e.g., alanine) is bound through a proper linkage to a solid-phase polymer to initiate the synthesis and serve as an internal standard. An inner core matrix is constructed from several levels of lysine to generate multiple reactive ends (two to eight ends) to which peptides can be linked. The surface layer is composed of either multiple copies of linear or cyclic synthetic peptides attached to the lysine inner-core matrix (Fig. 18.5.1). The MAP makes use of a limited sequential propagation of a trifunctional amino acid to form a low-molecular-weight core matrix. Lysine is suitable for this purpose because both N^{α}- and N^{ε}-amino groups are available as reactive ends. The sequential propagation of lysine generates $2n$ reactive ends, each of which can serve as an attachment site for peptides. The complete MAP has been shown to be large enough to stimulate immune responses.

The lysine core matrix is small; the bulk of the complex is formed by up to 16 peptides layered around the inner core matrix. As an example, a peptide dendrimer with an octabranched core matrix and eight copies of the peptide, each containing 14 amino acid residues, has a total molecular weight of 13 to 14 kDa. Only 7% of the mass is due to lysine residues of the core matrix; 93% of the mass is due to peptide sequences. The core matrix is oligomeric and contains noncationic peptidyl and isopeptidyl lysine amide on both N^{α}- and N^{ε}-termini of lysine. As a result, the MAP core matrix is totally bioinactive and functions only as a template to keep the multiple peptides together. It is the dendritic peptide chains on the core matrix that are likely to be mobile and contribute to the biological activity of the MAP system. Recently, Wrighton and co-workers (Wrighton et al., 1997) have used β-Ala-Lys as a pseudosymmetrical dendrimeric template and have attached two copies of the erythropoietin mimetic peptide (EMP) to the ε-amino group and the α-amino moiety, respectively. The two intramolecular disulfide bridges in EMP were formed by using an orthogonal protecting group scheme. As a result, the dimer has 100-times higher affinity for the erythropoietin receptor (EPOR) and 10-fold better antagonistic activity in vivo than the monomer.

The MAP system is chemically unambiguous, and the method of synthesis provides accurate knowledge of conformation and quantity of the peptide. The MAP system is also versatile. If half of the reactive lysine amino chains (i.e., ε-amino groups) are chemically blocked during synthesis, a peptide dendrimer containing two different peptide sequences can be constructed. For example, N^{α}- and N^{ε}-amino groups of lysine at the last level are orthogonally protected with *t*-butyloxycarbonyl (Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc), respectively. The first peptide chain can be assembled on the N^{α}-termini of this special lysine core matrix using Boc chemistry. The Fmoc groups of N^{ε}-termini are not affected during the Boc synthesis. After synthesis of the first peptide is complete, the second peptide chain can be synthesized on the N^{ε}-termini using Fmoc chemistry. The entire MAP system with two different peptide sequences is then cleaved from resin by hydrogen fluoride (HF) cleavage. One such MAP system was used as the model for synthetic hepatitis B vaccines (Tam and Lu, 1989). The simplicity and flexibility of the design and synthesis of MAP also permit incorporation of a hydrophobic built-in adjuvant such as tripalmitoyl-*S*-glyceryl cysteine (P3C; Defoort et al., 1992).

Constrained peptides that mimic native protein conformations are highly desirable because of the advantages in improving their biological activity. To achieve such constrained peptides, linear peptide sequences need to be circularized and then attached to a lysyl MAP core matrix. This approach requires two types of synthetic manipulations: a cyclization strat-

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egy of a linear precursor and a chemoselective method to attach to the template. The conventional approach to achieving two synthetic components is usually cumbersome and requires a scheme of multi-tiered protecting groups. For example, conventional cyclization to form cyclic peptides requires a protection scheme for selected functional side chains. The reactions are carried out in organic solvent (off-resin) or solid phase (on-resin). After cyclization, the side-chain protecting groups are removed. This unit describes two simplified approaches (the direct approach and the indirect approach; also see Fig. 18.5.2) to form cMAPs through unprotected constrained peptides generated from their linear unprotected peptides under aqueous conditions. The authors' two approaches do not use protecting groups and produce cMAPs directly for a variety of purposes, including antigen scanning and generation of constrained peptide libraries. The direct and indirect methods for preparing cMAP are the same as for MAP, and include an extra cyclization step after the linear unprotected precursors are synthesized.

Synthetic peptides have been used extensively for production of site-specific antibodies for confirming the identity of proteins derived from recombinant DNA, for exploring biosynthetic pathways, and for defining precursor-product relationships (e.g., proenzyme and preproenzyme) and structural domains of proteins (Lerner, 1982). So far, the MAP approach has been used in the preparation of experimental vaccines (Amon and Horwitz, 1992) against hepatitis (Tam and Lu, 1989), malaria (Tam et al., 1990; Pessi et al., 1991), foot-and-mouth disease (Francis et al., 1991), and HIV (Defoort et al., 1992; Nardelli et al., 1992).

Various templates derived from peptide and organic compounds containing several peptide chains have been able to induce protein structural motifs like α-helix, β-sheet, β-turn, and loop conformations (Schneider and Kelly, 1995). Since the MAP system also has an increased ordered helical structure (Shao and Tam, 1995) and its synthesis is well established, MAP is suitable as a template to mimic proteins. Many important questions, such as how a certain structural motif can be induced and how the secondary structures interact with each other, can be addressed by using the authors' MAP system. Artificial enzymes can also be designed and generated because certain tertiary structures associated with a specific enzymatic activity can be mimicked on the surface of the MAP core matrix.

Critical Parameters

Chemistry of synthesis

The MAP system can be effectively synthesized by both Boc and Fmoc chemistry (Tam and Spetzler, 1997). The selection of the synthetic method depends on several factors, including peptide sequence, instrumentation requirements, cost, and chemical wastes. The most important factor is probably peptide sequence. For sequences very rich in amino acids that are susceptible to strong acid modifications [e.g., alkylation (Cys, Met, Trp, and Tyr), acylation (Glu), and dehydration (Asp-Gly, Asp-Ser)], Fmoc chemistry may provide better results than Boc chemistry. However, use of the low-high hydrogen fluoride cleavage technique has greatly reduced these types of side reactions. Sequences rich in Arg, Trp, Tyr, and Met, as well as peptides with proline as one of the first two residues from the C-terminus, may do poorly in Fmoc chemistry. Occasionally, a peptide cannot be successfully prepared by either of these two methods. A failed synthesis may be caused by sequence-dependent side reactions.

The second factor that affects the choice of synthetic method is the instrumentation requirement. In general, Fmoc chemistry is easier in operation and requires less equipment. Boc chemistry normally needs special equipment for the final hydrogen fluoride cleavage of peptide from resin. If cost is a consideration, Boc chemistry is preferable because Boc-amino acid derivatives are less expensive than Fmoc-amino acid derivatives. The nature and quantity of chemical wastes produced during synthesis should also be considered. The solvent, coupling reagent, and cleavage reagent wastes generated in Boc chemistry are acidic (due to TFA) and contain halogens (TFA, DCM). The waste generated by Fmoc chemistry is basic (due to piperidine) and is not halogen-containing; the DCM used in reaction 8 of the procedure (Table 18.5.2) is negligible in this regard. For the synthesis of cMAPs, the preparation of their linear precursors are often limited to either Boc or Fmoc chemistry depending on the cyclization method used (see discussion of Method of Synthesis, below). However, the same precautions have to be taken as for the synthesis of MAP.

Method of synthesis

Both direct and indirect approaches for preparing MAPs are presented in this unit. In the direct approach, the linear peptide sequence

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and MAP core matrix are synthesized in a single operation, directly on the resin matrix, by stepwise solid-phase peptide synthesis (Basic Protocol 1 and the Alternate Protocol). The core matrix (two or three levels of lysine) is formed first by coupling lysine to a small amino acid attached to a resin support. The peptide sequence is then synthesized stepwise on this branched-lysine scaffolding. After peptide synthesis, the MAP is cleaved from the resin support. In the indirect approach, the MAP core matrix and peptide are synthesized separately, purified, and then combined to form the complete MAP by one of a number of different ligation methods illustrated in Figure 18.5.5 (Basic Protocols 2 to 3). One advantage of the indirect approach is that the components—MAP core matrix and peptide—are purified before conjugation. Any impurity generated during solid-phase peptide synthesis can be removed; this may be difficult in the direct approach because of the unique structural characteristic of MAP.

Preparation of cMAP using the direct approach can be complicated by competing intramolecular versus intermolecular cyclization. The competing reactions can be minimized for single-chain peptides under dilute conditions, but this is not possible for cMAPs because of simultaneous multiple peptide copies on the branched structure. Furthermore, extensive intermolecular cyclization among the precursors can also give polymers. The authors' solution for avoiding side products and for simultaneously obtaining a self-assembly process to give multiple cyclic peptide is based on ring-chain tautomerization. Ring-chain tautomerization is an entropy process and is conformation-driven as well as concentration-independent, to favor intra- over intermolecular reactions. The initial bond formation is reversible in ring-chain tautomerism. Thermodynamically stable products are formed under equilibrating conditions suitable for self-assembly of the multiple peptide chains on the dendrimer to form end-to-side chain cyclic peptides. In the synthetic scheme of cMAP, the ring-chain tautomerization for intramolecular cyclization is a thiazolidine ring generated from an N-terminal Cys and an aldehyde attached to the side chain of Lys (Fig. 18.5.6). Peptides with a motif that favor a cyclic structure are highly recommended in this approach.

The authors' schemes for preparing cyclic peptides by the indirect approach result in ligation sites of peptide bonds like X-Cys and X-SPro and surrogate peptide bonds such as thiazolidine and oxime that both are metabolically stable (Fig. 18.5.3). These schemes also produce peptides which are cyclized through end-to-end, end-to-side chain, or side chain to side chain (Pallin and Tam, 1995, 1996; Botti et al., 1996; Zhang and Tam, 1997), depending on the strategic placement of the two intended sites that must react with each other. Although peptide and nonpeptide bonds are described, the chemistries governing their syntheses are rather similar. To generate unprotected cyclic peptides with peptide bonds such as X-Cys and X-SPro from linear unprotected precursors, the reaction must be regioselective (Liu and Tam, 1994; Tam, 1995). A common strategy for obtaining regioselectivity is entropic activation achieved by capturing the N^{α}- and C^{α}-termini at close proximity as a covalent bond allowing an intramolecular acyl shift and forming a peptide bond. In cyclization reactions, the capturing reaction between an N^{α}-amine and a weakly activated C^{α}-acyl moiety has to be specific in the presence of many other side-chain functional groups, including N^{ε}-amine. Two methods are available to form a Cys bond in the ligation site. In one method, cyclization is achieved via an intramolecular transthioesterification of the linear precursor containing an N-terminal Cys and a C-terminal thioester. The thioester intermediate subsequently undergoes a proximity-driven S- to N-acyl transfer to give the desired amide bond, and introduces a thiol moiety (Basic Protocol 5). In the other method, the cyclization occurs via an acyl disulfide–mediated intramolecular acylation. The linear precursor consists of an N-terminal Cys protected and activated as Cys(Npys) and a C-terminal thiocarboxylic acid. The mixed disulfide undergoes an S to N-acyl transfer through a six-member ring and a native Cys residue is obtained after thiolytic reduction (Support Protocol 4). Cyclic peptides with a thiol moiety are useful as building blocks in the indirect approach for preparing cMAPs (see discussion of Method of Ligation, below). Also, cyclic peptides containing an SPro residue to mimic Pro can be generated (Support Protocol 2). The cyclization is mediated by an amine-aldehyde condensation. The linear precursor contains an N-terminal Cys and a C-terminal glycoaldehyde generated from $NaIO_4$ oxidation of the corresponding glyceric ester. The Cys residue is protected as Cys(StBu) because of its sensitivity to $NaIO_4$ oxidation. First, a thiazolidine ring is formed when the StBu protecting group is removed by phosphine. Then a proximity-driven *O* to *N*-acyl transfer reaction occurs through a tricyclic

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ring contraction to afford the peptide bond. Cyclic peptides through side-chain-to-end and end-to-end can be achieved.

In the preparation of cyclic peptides with peptide bond surrogates from totally unprotected linear precursors, chemoselective ligation based on carbonyl chemistry is used. Carbonyl chemistry exploits the selectivity of weak bases in the condensation with an aldehyde under acidic conditions where the basic side-chain nucleophiles are protected by protonation. For the purposes here, weak bases such as thiols, hydrazides, and amino-oxy groups are available, since they are easy to incorporate into the peptide during solid-phase synthesis. An aldehyde is obtained by $NaIO_4$ oxidation of N-terminal Ser, Thr, or Cys under neutral conditions to give an α-oxoacyl group. A thiazolidine ring is formed from the reaction between the aldehyde and the 1,2-aminoethanethiol of N-terminal Cys. Under the same conditions, an amino group will form a reversible Schiff base with the aldehyde. The Cys residue, which is incorporated as Cys(StBu), can play a dual function in the synthetic scheme. First, it can be used for cyclization where the cyclization is mediated after deprotection of the thiol of Cys, as already mentioned above. Second, it can be applied for ligating unprotected cyclic peptides to a core matrix when the Cys residue is deprotected after the cyclic peptide is formed. Another weak base is aminooxy, which affords oxime at low pH. In the authors' scheme, the aldehyde group is attached to the side chain of Lys while the nucleophilic aminooxyl or cysteinyl moiety is incorporated to either the amino terminal of the peptide or through the side chain of Lys to afford end-to-side chain or side chain-to-side chain cyclic peptides.

Method of ligation

The advantage of the indirect approach is that conjugation between the unprotected peptide and MAP core is chemically unambiguous. The functional groups on the peptide chain and core matrix can be designed to avoid reacting with other functional groups on the peptide. A variety of conjugation methods and cross-linking reagents may be used for conjugation to avoid modification of the peptide. The methods described in this unit are some of many variations (Fig. 18.5.5). A pair of mutually reactive functional groups such as a nucleophile and an electrophile are introduced on the monomeric peptide and MAP core matrix, respectively. The two functional groups only react specifically with each other. The methods that use thiol and carbonyl chemistries are suitable for the preparation of both MAP and cMAP with nonpeptide bonds.

A thiol group can be selectively reacted in the presence of amine and other side-chain groups because it is a stronger nucleophile, especially in neutral or acidic conditions where the amine group is protonated. Haloacetyl (Fig. 18.5.5) is a functional group used for selectively reacting with a thiol group under neutral to slightly basic pH conditions. However, under these conditions, the thiol tends to be oxidized to disulfide. Both unprotected linear (Basic Protocol 2) and cyclic (Basic Protocol 6) peptides contain a free thiol that has been ligated to chloroacetyl groups incorporated on the lysine core matrix. The indirect approach using thiol chemistry also allows the peptides to be attached to the MAP core matrix through either the amino or the carboxyl terminus, increasing the flexibility of the design. However, other functional groups such as maleimide or aromatic thiols like 2-thiopyridyl, nitropyridyl sulfenyl, or 4-dithiopyridyl, which also react specifically with a thiol, can be applied to ligate unprotected peptides to the MAP core matrix.

Carbonyl chemistry that exploits the selectivity of weak bases in condensation with an aldehyde has already been mentioned (see discussion of Method of Synthesis, above) and is shown in Figure 18.5.5. Thiazolidine ring formation has been used to ligate both linear (Basic Protocol 3) and constrained peptides (Basic Protocol 5) to a MAP core matrix containing α-oxoacyl groups. Other types of weak bases are hydroxylamine and hydrazine, which afford oxime and hydrazone bonds with an aldehyde moiety. Oxime and hydrazone bonds in the ligation site have been suitable for linear unprotected peptides (Basic Protocol 3). Both types of weak bases are added to the N-terminus, so the peptide sequence will be oriented with the amino terminal residues proximal to the core matrix and the carboxyl terminal residues distal to core matrix.

The conjugation reactions listed in Figure 18.5.5 have some limitations. The haloacetyl derivative may react with amine or other nucleophilic groups in peptides in basic medium. This type of reaction will lead to chemical ambiguity of the conjugate. To prevent this reaction, the pH of the conjugating solution should not be above 9.0. Aldehyde groups reacting with N-terminal cysteine rapidly form a stable thiazolidine derivative at pH 4 to 6. The resulting thiazolidine is quite stable under normal manipulating conditions, but the aldehyde

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itself is unstable. However, this can be circumvented by inert gas protection. Hydroxylamine and hydrazine react with aldehydes, forming an oxime and a hydrazone linkage, respectively, at pH ~5. Both linkages are stable at physiological pH (6 to 8), but the hydrazone derivative will be hydrolyzed at a lower or higher pH. The rate of hydrolysis is pH dependent. Stability of the linkage can be increased by reducing the hydrazone bond to hydrazine with sodium cyanoborohydride ($NaBH_3CN$).

Troubleshooting

After the peptide sequence is selected, MAP or cMAP with this special sequence can be synthesized by solid-phase synthesis. As with solid-phase peptide synthesis, the reactions of each cycle (including deblocking and coupling reactions) must be very efficient and should be driven to completion in a short time. The following calculations demonstrate the importance of high yield from each cycle. If the mean yield of each cycle of synthesis is 95%, the final yield for a peptide with 20 amino acid residues would be only 35.8%. However, if the yield of each cycle is 98%, 99%, 99.5%, or 99.9%, the total yields for the synthesis of a peptide with 20 residues would be 66.8%, 81.8%, 90.5%, or 98.0%, respectively. Furthermore, the byproducts produced in peptide synthesis usually closely resemble the desired peptide product in sequence and structure and are very difficult to remove by normal separation methods. Because our MAP system is a dendrimer, the degree of heterogeneity of peptide products will be much higher than that of solid-phase peptide synthesis. For example, in the synthesis of a MAP system with four branches, a 99% yield from each cycle results in 96% of MAP with the correct structure. If the mean yield of each cycle of synthesis is 99%, the synthesis of a four-branched MAP system with a peptide antigen of 20 amino acid residues will result in final yield of 44.2% with the correct sequence, instead of 81.8% obtained in usual solid-phase peptide synthesis.

The major side reactions during solid-phase peptide synthesis are: deletion, where one or more amino acid residues are skipped during synthesis; termination, where chain elongation stops at a point because of acylation or other side reaction; and racemization, where the amino acid is racemized during peptide elongation. All these side reactions result in heterogeneity of peptide product, and decrease the total yield of synthesis. To overcome these side reactions, excess amounts of reagents should be used in the synthesis to force reactions to completion and to shorten the reaction time. Double coupling or even coupling at elevated temperatures (50° to 60°C) should be used if a single coupling fails to complete the reaction.

Efficiency of synthesis should be closely monitored by the ninhydrin test (Support Protocol 1) or quantitative ninhydrin test (Sarin et al., 1981). The unusual properties of MAP systems (e.g., their multibranched structure and tendency to aggregate) should be considered to achieve a high-quality product. The synthesis should be started with a lower level of amino acid loading on the resin than is customary with usual solid-phase peptide synthesis, because peptide content will increase geometrically with each additional layer of lysine. Peptide synthesis will be difficult if the conventional loading of 0.3 to 0.8 mmol amino acid/g resin is used. In the Alternate Protocol for Fmoc chemistry, because resin with 0.1 mmol/g loading is not commercially available, synthesis starts with a resin of 0.3 to 0.5 mmol alanine/g resin, but only some of the amino groups are used for preparation of the dendrimer. Dimethyl formamide (DMF) is a more suitable solvent than dichloromethane (DCM) for avoiding potential aggregation of peptides. The peptide resin should not be dried at any stage of synthesis because resolvation of dried resin that contains MAP scaffolding is difficult.

During HF cleavage in Boc synthesis, several precautions should be taken to obtain satisfactory results. The HF line must be kept clean. The line should be checked before each cleavage. Very often, heterogeneous and complex peptide products are caused by a dirty, contaminated HF line. The HF line should also be checked for leaks before each cleavage. A leaky HF line can result in the loss of HF and dimethyl sulfide (DMS) during the cleavage reaction; this can lead to unsatisfactory results. A leaky HF line may also allow water and carbon dioxide into the reaction vessel, diluting the HF cleavage mixture. The cleavage mixture should be mixed completely during the reaction. Sometimes the magnetic stir-bar is frozen by the *p*-cresol or *p*-thiocresol mixture during the introduction of the reagent. This can be avoided by adding the reagents in the following order: (1) peptide resin, (2) *p*-cresol and *p*-thiocresol in melted form, then, after cooling and when the mixture has solidified, (3) the magnetic stirbar, and (4) DMS.

In Fmoc synthesis, trifluoroacetic acid (TFA) cleavage should be prolonged if the peptide contains Arg(Pmc). Thioanisole can

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accelerate the cleavage of the Pmc group. The deprotection of trityl (Trt) from cysteine residues is reversible, and scavengers such as thiophenol and 1,2-ethanedithiol (EDT) should be added to the cleavage mixture to prevent Trt reattachment to Cys. EDT also minimizes the alkylation reaction of Trp. The optimal volume of cleavage solution should be determined experimentally. In general, larger volumes of cleavage solution will, to some extent, suppress side reactions because most of the side reactions in the cleavage stage are bimolecular reactions. However, the more cleavage solution used, the larger the volume of TFA that must be removed, and the larger the amount of scavenger that will remain, making purification and analysis of the peptide more difficult. TFA can be reduced to a smaller volume under a vacuum by means of rotary evaporation, but the mixture should never be dried. A small-scale cleavage, using 20 to 30 mg of peptide resin, should be performed to determine optimal cleavage reaction conditions such as the combination and the ratio of scavengers, reaction time, and the volume of cleavage mixture. HPLC can be used to evaluate the results. Reaction conditions should be modified if the crude cleavage product is not as homogeneous as expected. The desired degree of purity should be ~60% or a major peak in the HPLC.

MAP systems prepared by the direct approach have been found to have an unusual tendency to aggregate after cleavage from the resin. The entire MAP should be dialyzed under basic, denaturing conditions using 8 M urea to remove unwanted additives from the cleavage reaction. Treatment with base under these conditions will also convert the possible strong acid–catalyzed *O*-acyl rearrangement product of serinyl and threonyl peptides back to *N*-acyl peptides. After dialysis, MAP systems can be further purified by either RP-HPLC or high-performance gel-filtration chromatography.

In the additional cyclization step for preparing cyclic unprotected peptide building blocks and the entire cMAP by the direct approach, some of the protocols involve oxidation with $NaIO_4$. The order of the synthetic scheme to generate aldehydes from the oxidation has to be followed carefully, since not all the functional groups in their unprotected form, suitable for cyclization, are stable under the condition of $NaIO_4$. Perform the $NaIO_4$ oxidation at neutral pH in the presence of a large excess of free methionine as a scavenger, to prevent oxidation of Met to Met(O). The oxidation rate increases as pH decreases, but side reactions occur at lower pH. The aldehyde may decompose, and the absence of air is necessary. Flushing with nitrogen or argon for 30 min results in an oxygen-free solution. For cyclizations that include the presence of free sulfhydryl groups, the reactions must also be carried out in a solution devoid of oxygen to avoid dimerization. Also, addition of thiols or trialkylphosphine to the solution prevents the formation of dimers. The cyclization reactions can be carried out in a final concentration of up to 20 mM without affecting the yield of the desired product, since the reactions usually are concentration-independent.

In the indirect approach where either linear or cyclic unprotected peptides are ligated to a MAP core matrix using carbonyl or thiol chemistry, the ligation reactions are also performed in an oxygen-free solution. Usually, the ligation is performed with a peptide concentration at 1.2- to 2-fold excess relative to core matrix. For the thiol alkylation reaction in thiol chemistry, iodoacetylated and bromoacetylated derivatives are known to be more reactive than the chloroacetylated derivative. However, the chloroacetylated derivative is more stable during the preparation of core matrix, especially during HF cleavage.

The peptide and nonpeptide bonds formed in MAP and cMAP are stable at physiological pH (pH 6 to 8) as well as at low pH during analytical and preparative HPLC. However, for long-term storage, the peptides must be stored as a dry powder at a maximum temperature of –20°C.

Anticipated Results

If synthesis of an eight-branched MAP with ~15 amino acid residues starts from 0.5 g resin with a resin loading of 0.1 mmol/g, then >0.5 g of crude MAP can be obtained. Its purity depends on both the peptide sequence and the method used for synthesis. Usually the purity of crude products will be >60%, and after purification ~40% to 50% can be recovered as purified MAP. This amount is usually more than sufficient for biological purposes. In general, the reaction should start with 0.3 to 0.5 g resin. For preparing cMAP using the direct approach, the overall yield can be up to 60% for cyclic peptides containing 17 and 24 amino acid residues, also starting with 0.5 g resin (loading is 0.1 mmol/g). In the direct approach, the average yield for MAP is between 50% and 60% using the ligation methods in Figure

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18.5.5. The yield of the pure cMAP system is ~30% when cyclic peptides ranging from 6 to 14 amino acids are used. However, the site and structure, as well as the excess and concentration of the peptides influence the obtained yield. Both methods for generating MAP and cMAP systems are applicable for mimetic proteins.

If HPLC of a crude MAP prepared by the direct approach shows multiple peaks without a major product, the complex may be difficult to purify, and the yield of MAP may not be satisfactory. In this case, the synthetic procedure (synthetic chemistry, protecting combination, coupling procedure, and/or cleavage conditions) should be modified. Also, the progress of the ligation reactions in the indirect approach is conveniently monitored by RP-HPLC (*UNIT 11.6*). If no product is formed, the peptides should be reanalyzed by mass spectrometry (Chapter 16) for the presence of nucleophilic and electrophilic moieties that have to react with each other. The peptide may even have to be resynthesized. Also, be aware of the formation of byproducts that can be generated from hydrolysis of the starting material at a given pH. Therefore, recheck the pH during the cyclization and assembling reactions.

Time Considerations

An automatic peptide synthesizer can assemble ~15 residues in one day by a rapid procedure using a 15-min cycle or in 2 days with the the normal cycle of ~1 hr. The synthesis of a MAP system with its peptide chain of 10 to 20 residues will usually take 1 to 2 days using the direct approach. Using a manual shaker for solid-phase peptide synthesis, assembly of as many as 6 to 12 amino acid residues onto the resin in one day is possible. The same MAP system can be prepared within 4 days by manual synthesis. Cleavage and subsequent purification can take from 2 days to 1 week, depending on the purity of the crude product as well as the required purity of the isolated MAP system. In preparing cMAP using the direct approach, 5 days should be added for the extra steps of cyclization, purification and characterization.

In the indirect approach for preparing MAP systems, both the peptide and core matrix are prepared and purified before ligation. Ligation of the peptide with the core matrix requires an additional reaction step and adds 2 to 3 days to the process. For cyclization of the linear unprotected precursors, 3 extra days are needed.

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IDENTIFICATION OF PROTEIN INTERACTIONS

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CHAPTER 19
Identification of Protein Interactions

INTRODUCTION

Protein interactions are intrinsic and vital to many cellular processes. In recent years many new techniques have been developed for the study of this important topic; the review by Phizicky and Fields (1995) provides an excellent general overview of this subject. This chapter describes biochemical, physical, and genetic methods that are used to detect specific protein interactions. A companion chapter in preparation will present a series of complementary methods that are used to provide quantitative information about protein interactions, such as the strength and kinetics of binding.

This chapter begins with brief overview of the analysis of protein interactions in which some of the basic kinetic and thermodynamic concepts are described (*UNIT 19.1*). This is followed by a presentation of one of the most widely used approaches for detecting protein interactions, the yeast two-hybrid/interaction trap system (*UNIT 19.2*). In this genetic method transcriptional activity is used as a measure of protein interaction.

In the technique of co-immunoprecipitation (*UNIT 19.4*), cell-free extracts are incubated with an antibody to a protein or an epitope tag in order to coprecipitate associated proteins. This approach is used to isolate multiprotein complexes that presumably were present in the intact cell.

In the technique of co-immunoprecipitation (*UNIT 19.4*), cell-free extracts are incubated with an antibody to a protein or an epitope tag in order to coprecipitate associated proteins. This approach is used to isolate multiprotein complexes that presumably were present in the intact cell.

Future plans for this chapter include units on a phage-based expression cloning system used to identify interacting proteins, and surface plasmon resonance (SPR), another useful method for discovery and detection of protein interactions that is gaining in popularity.

LITERATURE CITED

Phizicky, E.M. and Fields, S. 1995. Protein-protein interactions: Methods for detection and analysis. *Microbiol. Rev.* 59: 94-123.

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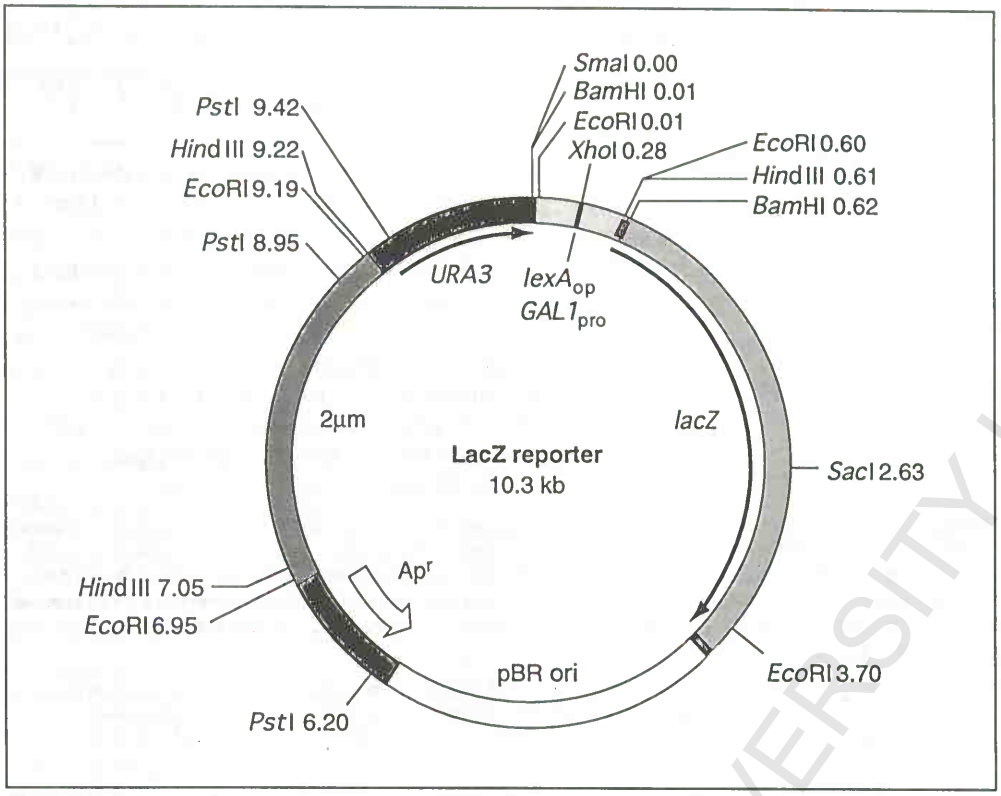

Figure 19.2.4 LacZ reporter plasmid. pRB1840, pJK103, and pSH18-34 are all derivatives of LR1Δ1 (West et al., 1984) containing eight, two, or one operator for LexA (*LexA*$_{op}$) binding inserted into the unique *Xho*I site located in the minimal *GAL1* promoter (*GAL1*$_{pro}$; 0.28 on map). The plasmid contains the *URA3* selectable marker, the 2µm origin to allow propagation in yeast, the ampicillin resistance (Ap^r) gene, and the pBR322 origin (ori) to allow propagation in *E. coli*. Numbers indicate relative map positions. In the recently developed derivatives, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol or kanamycin resistance genes (see Table 19.2.1 for details).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

Transform yeast with the bait protein plasmid

1. Using standard subcloning techniques (e.g., Struhl, 1987), insert the DNA encoding the protein of interest into the polylinker of pEG202 (see Fig. 19.2.3) or other LexA fusion plasmid to make an in-frame protein fusion.

   The LexA fusion protein is expressed from the strong alcohol dehydrogenase (ADH) promoter. pEG202 also contains a HIS3 selectable marker and a 2µm origin for propagation in yeast. pEG202 with the DNA encoding the protein of interest inserted is designated pBait. Uses of alternative LexA fusion plasmids are described in Background Information.

2. Perform three separate lithium acetate transformations (*APPENDIX 4L*) of EGY48 using the following combinations of plasmids:

   pBait + pSH18-34 (test)
   pSH17-4 + pSH18-34 (positive control for activation)
   pRFHM1 + pSH18-34 (negative control for activation).

   Use of the two LexA fusions as positive and negative controls allows a rough assessment of the transcriptional activation profile of LexA bait proteins. pEG202 itself is not a good negative control because the peptide encoded by the uninterrupted polylinker sequences is itself capable of very weakly activating transcription.

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Table 19.2.2 Interaction Trap Yeast Selection Strains[a]

Strain	Relevant genotype	Number of operators	Comments/description
EGY48[b,c,d]	*MATα trp1*, *his3*, *ura3*, lexAops-LEU2	6	*lexA* operators direct transcription from the *LEU2* gene; EGY48 is a basic strain used to select for interacting clones from a cDNA library
EGY191	*MATα trp1*, *his3*, *ura3*, lexAops-LEU2	2	EGY191 provides a more stringent selection than EGY48, producing lower background with baits with instrinsic ability to activate transcription
L40[c]	MATα *trpl*, *leu2*, *ade2*, GAL4, *lexAops-HIS34*, *lexAops-lacZ8*		Expression driven from *GAL1* promoter is constitutive in L40 (inducible in EGY strains); selection is for *HIS* prototrophy. Integrated *lacZ* reporter is considerably less sensitive than pSH18-34 maintained in EGY strains

[a]Interaction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (EGY48, EGY191); A.B. Vojtek and S.M. Hollenberg, Fred Hutchinson Cancer Research Center, Seattle, Wash. (L40). Specialized strains not yet commercially available can be obtained by contacting the Brent laboratory at The Molecular Sciences Institute, Berkeley, (510) 647-0690 or *brent@molsci.org*, or the Golemis laboratory, (215) 728-2860 or *EA_Golemis@fccc.edu*.

[b]Strains commercially available from Clontech.

[c]Strains commercially available from Invitrogen as components of a Hybrid Hunter kit; the kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

[d]Strains commercially available from OriGene.

pSH18-34 contains a 2μm origin and a URA3 selectable marker for maintenance in yeast, as well as a bacterial origin of replication and ampicillin-resistance gene. It is the most sensitive lacZ reporter available and will detect any potential ability to activate lacZ transcription. pSH17-4 is a HIS3 2μm plasmid encoding LexA fused to the activation domain of the yeast activator protein GAL4. This fusion protein strongly activates transcription. pRFHM1 is a HIS3 2μm plasmid encoding LexA fused to the N-terminus of the Drosophila protein bicoid. This fusion protein has no ability to activate transcription.

3. Plate each transformation mixture on Glu/CM –Ura, –His dropout plates. Incubate 2 days at 30°C to select for yeast that contain both plasmids.

   Colonies obtained can be used simultaneously in tests for the activation of lacZ (steps 4 to 7) and LEU2 (steps 12 to 13) reporters.

Assay lacZ gene activation by β-galactosidase assay

4. Streak a Glu/CM –Ura, –His master dropout plate with at least five or six independent colonies obtained from each of the three transformations in step 3 (test, positive control, and negative control) and incubate overnight at 30°C.

   The filter assay described in Steps 5a to 7a (based on Breeden and Nasmyth, 1985) provides a rapid assay for β-galactosidase transcription. Alternatively, a liquid assay (APPENDIX 4L) or a plate assay (described in Steps 5b to 7b) may be used.

Perform filter assay for β-galactosidase activity:

5a. Lift colonies by gently placing a nylon membrane on the yeast plate and allowing it to become wet through. Remove the membrane and air dry 5 min. Chill the membrane, colony side up, 10 min at –70°C.

   Whatman 3MM filters can be cut to the size of the yeast plate as a more economical alternative to nylon membranes for performing lifts. In addition, two or three 5-min temperature cycles (–70°C to room temperature) can be used instead of a single cycle to promote better lysis; this may be worth doing if there is difficulty visualizing blue color.

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Detection of Protein-Protein Interactions by Coprecipitation

Coprecipitation of proteins from whole-cell extracts is a valuable approach to test for physical interactions between proteins of interest. When a precipitating antibody is used, this method is referred to as co-immunoprecipitation. Coprecipitation can be used to study interactions between known proteins under a variety of conditions and as a means of identifying components of a complex. Coprecipitation may be the single method of choice, or may be used in combination with other methods that detect protein-protein interactions, such as two-hybrid analysis and copurification schemes (*UNIT 19.2*), and tests of physical associations using purified proteins.

This unit describes basic approaches to immunoprecipitating tagged proteins from whole-cell extracts. The approaches described can be adapted for other systems. In a typical experiment, as described here, cells are lysed and a whole-cell extract is prepared under nondenaturing conditions (see Strategic Planning). The protein is precipitated from the lysate with a solid-phase affinity matrix and the precipitate is tested for the presence of a second specifically associated protein (see Basic Protocol and Alternate Protocol). The approach can be used for native or epitope-tagged proteins for which antibodies are available, or for recombinant proteins that have been engineered to bind with high affinity to a molecule that can be coupled to a solid-phase matrix (see Strategic Planning). The presence of an associated protein is detected by separating the precipitated proteins by SDS-PAGE (*UNIT 10.1*) and then immunoblotting (*UNIT 10.10*) with a second antibody that recognizes the putative associated protein. Controls to test specificity of interaction are crucial (see Strategic Planning).

For additional background reading, the user should consult *UNIT 3.8* for a theoretical discussion of immunoprecipitation and Coligan et al. (1999) for principles of antibody production and immunoassays; see *UNIT 19.3* for approaches to tagging proteins; and see *APPENDIX 4L* and *UNITS 5.6-5.8* for transformation and propagation of yeast. For an in-depth review of immunoprecipitation techniques, see Chapter 11 of Harlow and Lane (1988). For an in-depth review of coprecipitation and other approaches to detect protein-protein interactions, see Phizicky and Fields (1995).

STRATEGIC PLANNING

Detecting the Proteins in Question

The first step is to generate reagents that detect the two proteins in the coprecipitate under nondenaturing conditions. If antibodies are available that can immunoprecipitate the proteins under nondenaturing conditions, then these can be used. Alternatively, the proteins can be differentially tagged in a variety of ways to allow their detection with commercially available antibodies or other affinity reagents. The tagged proteins are then introduced into the host organism using expression vectors. All tagged proteins must be assessed for function in vivo.

A frequently used option is to add a short peptide or epitope that is recognized by a commercially available high-affinity monoclonal antibody (mAb). The epitope is typically added at the amino or carboxyl terminus, although internal positions that do not disrupt function can also be used. Two frequently used epitopes are derived from influenza hemagglutinin protein (HA) and human c-Myc and are recognized by high-affinity mAbs (12CA5 and 9E10, respectively; Kolodziej and Young, 1991). Others, such as FLAG, are

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also available (BioSupplyNet Source Book, 1999). The choice of epitope may be dictated by its amino acid composition. It is often useful to insert tandem copies of the epitope in order to increase sensitivity. The number of additional tandem copies can range widely, from one (Field et al., 1988) to several (e.g., three; Tyers et al., 1993) to many (e.g., nine; Feng et al., 1998).

Proteins can also be fused to small proteins or peptides that have high affinity to small molecules that can be attached to a solid support. This is a particularly valuable approach when the protein to be precipitated comigrates with immunoglobulin heavy or light chains in an SDS-polyacrylamide gel. Such alternative tagging methods include fusion to glutathione-*S*-transferase (to allow purification by a glutathione affinity matrix; *UNIT 6.6*) or maltose-binding protein (to allow purification by a maltose affinity matrix; *UNIT 5.1*). An excellent reference for identifying sources of commercially available antibodies and approaches to tagging proteins is the BioSupplyNet Source Book (1999). See Coligan et al. (1999) and Harlow and Lane (1988) for the generation and purification of specific antibodies. See Chapters 5, 6, and 7 of this manual for a discussion of tagging and expressing proteins.

Preparing Whole-Cell Extracts

The second step in a successful coprecipitation is generating whole-cell extracts that optimize the yield and activity of the proteins to be analyzed, using lysis buffer conditions that permit recognition of the proteins by the affinity matrix. The yield of total protein in a whole-cell extract is not always a reliable indicator of the relative yield and activity of specific proteins, so it is wise to verify both parameters at the onset of an experiment before proceeding with the coprecipitation. Yield and activity can be affected by a number of factors (see Chapter 10; see Harlow and Lane, 1988). Small variations in the relative amounts of salt and detergents in the lysis buffer can have large effects on yield and activity, as can the speed and efficiency of cell breakage. Both factors are particularly important for less soluble proteins that associate with macromolecular structures such as membranes or cytoskeleton. In addition, global inhibition of proteolysis through the inclusion of multiple classes of protease inhibitors may be essential.

Methods for preparing whole-cell extracts from yeast (*UNITS 5.6-5.8*), *Escherichia coli* (*UNIT 6.2*), insect cells (*UNIT 5.12*), and mammalian cells (*UNITS 5.9 & 5.10*) can be found elsewhere in this manual, and specifics will not be discussed here. In general, the lysis buffer conditions are not very different from the coprecipitation conditions. It is recommended that the investigator begin by comparing small-scale extract preparations that vary the amount of salt and nonionic detergent. As a starting point, a basic lysis buffer might contain the following components.

Basic components. Basic components include a buffering agent (such as 50 mM Tris·Cl, pH 7.5), a small amount of nonionic detergent (such as 0.1% [v/v] Triton X-100), salt (such as 100 mM NaCl), a reducing agent (such as 1 mM DTT), and 10% (v/v) glycerol as stabilizer.

Protease inhibitors. Protease inhibitor cocktails are described here in *UNIT 5.8* and are also commercially available. A reasonable starting point would be to include 5 µg/ml each of chymostatin, pepstatin A, leupeptin, and antipain, as well as 1 mM phenylmethysulfonyl fluoride and 1 mM benzamidine.

Chelating agents. EGTA (~15 mM) is commonly included to chelate divalent metal ions that are essential for metalloprotease activity. Because EGTA also inhibits other metal-dependent enzymes, it may be omitted, combined with the addition of a needed metal ion, and/or substituted with EDTA.

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Phosphatase inhibitors. If the phosphorylation state of the proteins in question is important, a mixture of phosphatase inhibitors should also be included in the lysis buffer. A starting mixture could contain 2.5 mM each meta- and ortho-vanadate, 10 mM NaF, and 10 mM β-glycerol phosphate.

Simple modifications of this initial buffer include varying the amount of NaCl (from 0 to 500 mM) and of Triton X-100 (from 0% to 1%). The investigator may choose to compare different means of breaking the cells (for example, glass-bead breakage versus liquid nitrogen/grinding methods for yeast cells; *UNITS 5.6-5.8*).

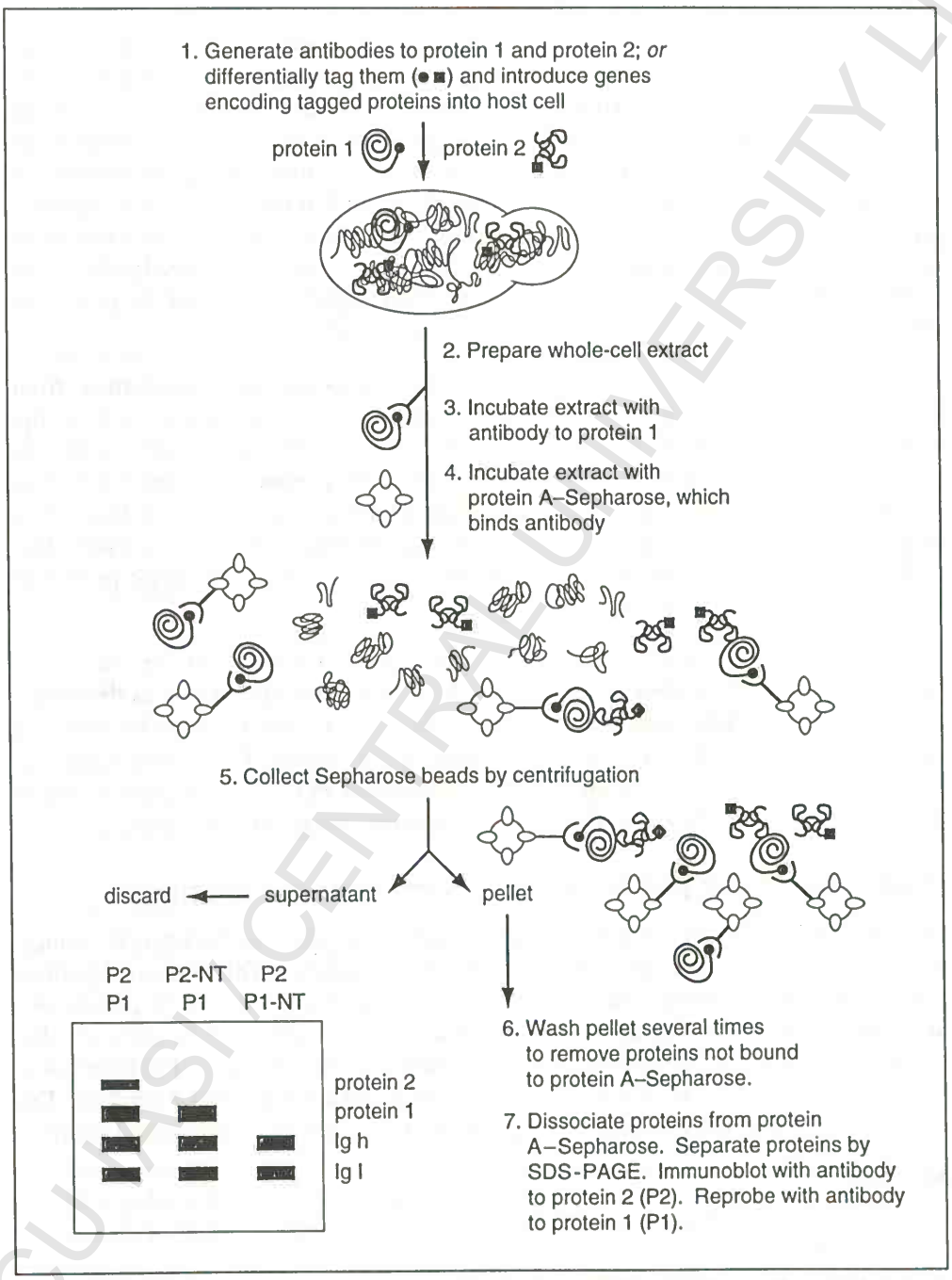

Figure 19.4.1 Flow chart for the coprecipitation of two proteins that have been differentially tagged and introduced into the host organism. Ig h and Ig l, immunoglobulin heavy and light chains; NT, no tag.

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Total protein concentration in the whole-cell extract is generally assayed by using the Bio-Rad protein assay and calculating protein concentration (*UNIT 3.4*). Extracts should be tested for the amount of each specific protein by immunoblot analysis (*UNIT 10.10*), analyzing 25 to 75 μg of total protein. In general, it is best to test for the presence of a second established protein (such as a housekeeping enzyme, cytoskeletal or ribosomal protein, or a previously defined component in the pathway being studied) as an internal control for normalization and as a positive control for the immunoblot. The amount of specific protein in the whole-cell extract is compared to the amount that is recovered by precipitation with an affinity matrix.

Control Tests for Specificity of Interaction

Controls are essential to verify that the antibodies and protein-protein interactions are specific. Proper controls are simplest to set up when the proteins are differentially tagged. In this instance, two parallel extracts are prepared from strains that contain each protein lacking the tag in the presence of the second tagged protein. An example is shown in the idealized gel in Figure 19.4.1, which includes lanes containing untagged protein 1 + tagged protein 2 and tagged protein 1 + untagged protein 2. If the antibodies are specific, untagged protein 1 will not immunoprecipitate. The presence of untagged protein 1 in the immunoprecipitate will indicate that it binds the affinity matrix nonspecifically. If the interaction between proteins 1 and 2 is specific, then tagged protein 2 will be present in the immunoprecipitate of tagged protein 1, but not in its absence.

If antibodies to native proteins are used, it is necessary to compare extracts made from strains harboring deletions of the proteins in question to test for the specificity of the antibody and the interaction. However, this is obviously possible only if the deletions do not cause inviability. If deletion mutations cannot be used, a common approach is to show that the preimmune serum or an antibody not known to be specific to either of the proteins in question does not coprecipitate them in a parallel experiment. However, the latter two controls do not rule out the possibility that the antibody is precipitating the protein in question through an indirect association.

It is also essential to compare the amount of coprecipitated protein with the amounts of the two proteins in question in the whole-cell extract. This allows one to determine whether apparent differences in the ability of the two proteins to coprecipitate are a secondary consequence of the relative abundance of the proteins. This control is particularly important when an interaction has been established and the investigator wishes to search for regulatory changes in association apart from changes in abundance.

BASIC PROTOCOL

COPRECIPITATING PROTEINS WITH PROTEIN A/G–SEPHAROSE

Once the conditions of extract preparation have been established (see Strategic Planning), the next step is to test for coprecipitation of the specific proteins. This protocol describes a standard coprecipitation procedure that uses an antibody coupled to protein A–Sepharose or protein G–Sepharose. An alternative coprecipitation method that uses GST coupled to glutathione-agarose is also provided (see Alternate Protocol). It is essential to keep all buffers and tubes cold by using an ice bath and a refrigerated centrifuge. The conditions of coprecipitation match the conditions of the lysis buffer described above.

Materials

Whole-cell extract (see Strategic Planning)
Antibody
Co-immunoprecipitation buffer (see recipe)
5 M NaCl (*APPENDIX 2E*)

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Protein A/G–Sepharose slurry (see recipe)
2× sample buffer for SDS-PAGE (*UNIT 10.1*)

Test tube rotator
20-ml syringe and 18-G needle
Hamilton syringe

Additional reagents and equipment for SDS-PAGE (*UNIT 10.1*) and immunoblotting (*UNIT 10.10*)

1. Prepare duplicate samples in microcentrifuge tubes on ice:

   0.5 to 1 mg whole-cell extract
   1 µg antibody
   5 M NaCl to equalize at 100 mM NaCl
   Co-immunoprecipitation buffer to 0.5 ml final volume.

   Adjust buffer if necessary for activity of the protein in question (see Strategic Planning, discussion of chelating agents).

2. Invert tube gently several times and incubate on ice for 90 min with occasional tube inversion.

   It is recommended that the investigator begin with a 90-min incubation. However, this incubation step can be shortened or lengthened.

3. Microcentrifuge 10 min at maximum speed, 4°C, to pellet nonspecific aggregates. Transfer supernatant to a new microcentrifuge tube.

4. Add 50 µl of protein A– or protein G–Sepharose slurry (25 to 30 µl bead volume). Be sure to evenly suspend the slurry before distributing it to the samples.

   Protein A has been used more frequently for historical reasons; however, protein G binds a broader range of Ig subtypes at higher efficiency.

5. Rotate tube gently at 4°C for 30 to 60 min.

   Rocking is much less efficient and should be avoided.

6. Gently pellet protein A/G–Sepharose by centrifuging 30 sec at 1000 rpm in a tabletop centrifuge, 4°C.

7. Wash pellet three times with 1 ml co-immunoprecipitation buffer. For each wash, gently invert tube three times before pelleting. After each pelleting, use a 20-ml syringe with an 18-G needle to aspirate and remove supernatant.

   It may be possible to omit the costly protease inhibitors from the buffer at this stage, but this has not been attempted to date.

8. Aspirate as much liquid as possible from the final without touching the beads and add 25 µl of 2× sample buffer.

   If desired, samples containing sample buffer can be frozen up to several months at –80°C prior to SDS-PAGE. In this case the buffer should be prepared with sterile stock solutions and made with 1 mM sodium azide included.

9. Prepare for SDS-PAGE analysis by boiling for 5 min, vortexing, and microcentrifuging briefly to pellet beads. Use a Hamilton syringe to load eluates onto an SDS-polyacrylamide gel, arranging duplicate samples to allow preparation of duplicate blots. Separate by electrophoresis (*UNIT 10.1*).

   A Hamilton syringe works well to remove the eluate from the beads during loading.

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10. Immunoblot duplicate samples separately with antibodies for each of the two proteins (*UNIT 10.10*). Be sure to include aliquots of the whole-cell extract for comparison and as a positive control for the immunoblot.

Each immunoblot can be reprobed with the antibody to the other protein.

ALTERNATE PROTOCOL

COPRECIPITATING A GST FUSION PROTEIN

GST fusion proteins may be coprecipitated by following the co-immunoprecipitation procedure (see Basic Protocol) with the modifications outlined below. This procedure might be used when the protein in question comigrates with immunoglobulin heavy or light chain in an SDS-PAGE gel or if the antibodies being used precipitate too many cross-reacting proteins, such as the protein being tested for association. Furthermore, it is possible to dissociate the purified GST fusion from the solid-state glutathione resin under gentle conditions through the use of imidazole. The approach is also useful in that it will increase the size of the protein sufficiently that this size increase can be used as a diagnostic feature in analyzing complexes.

Additional Materials (also see Basic Protocol)

Glutathione-agarose or glutathione-Sepharose slurry (see recipe)

1. Prepare duplicate samples as described for protein A/G–Sepharose (see Basic Protocol, step 1), omitting antibody.
2. Microcentrifuge 10 min at maximum speed, 4°C, to pellet nonspecific aggregates. Transfer supernatant to new microcentrifuge tube.
3. Add 30 µl glutathione-agarose or glutathione-Sepharose slurry (25- to 30-µl bead volume). Be sure to evenly suspend the slurry before distributing it to the samples.
4. Rotate the sample, pellet and wash glutathione-agarose/Sepharose, and perform SDS-PAGE and immunoblot analysis (see Basic Protocol, steps 5 to 10).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2E; for suppliers, see SUPPLIERS APPENDIX.

Co-immunoprecipitation buffer

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)
15 mM EGTA
100 mM NaCl
0.1% (w/v) Triton X-100
Store at 4°C
Immediately before use add:
1× protease inhibitor mix (see recipe)
1 mM dithiothreitol (DTT)
1 mM phenylmethylsulfonyl fluoride (PMSF; from fresh 250 mM solution in 95% ethanol)

If necessary for activity of the protein of interest, a divalent cation may need to be included in the buffer and EGTA omitted (as for extraction buffer; see Strategic Planning, discussion of chelating agents).

The protease inhibitor mix, PMSF, and DTT should be added fresh at the time of experimentation. The mixture without those components can be stored for months at 4°C with the addition of 1 mM sodium azide. PMSF is labile in aqueous buffer and should be added at the last minute.

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Glutathione-agarose or glutathione-Sepharose slurry

Swell 1.5 g glutathione-agarose or glutathione-Sepharose beads (e.g., Pierce, Sigma) in 30 ml of 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*), for 1 to 2 hr on ice. Pellet beads by gravity or very gentle centrifugation (1 min at 1000 rpm in a tabletop centrifuge) and then wash four times with co-immunoprecipitation buffer (see recipe) that lacks protease inhibitor mix and contains 1 mM sodium azide. Resuspend beads in 15 ml of this buffer to yield a final slurry concentration of ~100 mg/ml. Store at 4°C (stable for months).

This recipe can be scaled up or down.

Protease inhibitor mix, 1000×

Dissolve in DMSO:
5 mg/ml chymostatin
5 mg/ml pepstatin A
5 mg/ml leupeptin
5 mg/ml antipain
Store in aliquots up to 1 year at –20°C

Protein A/G–Sepharose slurry

Swell 1.5 g protein A– or protein G–Sepharose beads (e.g., Pierce, Sigma) in 30 ml of 50 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*), for 1 to 2 hr on ice. Pellet beads by gravity or very gentle centrifugation (1 min at 1000 rpm in a tabletop centrifuge) and then wash four times with co-immunoprecipitation buffer (see recipe) that lacks protease inhibitor mix and contains 1 mM sodium azide. Resuspend beads in 15 ml of this buffer to yield a final slurry concentration of ~100 mg/ml. Store at 4°C (stable for months).

This recipe can be scaled up or down.

COMMENTARY

Background Information

Coprecipitation is a powerful and simple approach to test for a physical interaction between proteins. There are many reasons to incorporate coprecipitation into a study. First, as a form of protein affinity chromatography, the method may be sensitive enough to detect weak associations that do not withstand the rigors of standard purification methods involving substantial dilution of the initial cell extract. Second, coprecipitation tests for associations between proteins within the milieu of a whole-cell extract, where the proteins are present at native concentration in a complex mixture of other cellular components. This feature makes it an important partner to two-hybrid methods and direct tests of interactions using purified proteins, because it provides a way to verify that a positive interaction reflects a true in vivo association. For example, nonphysiological interactions can be detected when purified proteins are present at too elevated a concentration. A falsely positive interaction between two proteins can also arise in a two-hybrid test when protein domains are inappropriately exposed due to altered folding. In addition, not all proteins are amenable to two-hybrid analysis; a negative result may mask a true association.

Nevertheless, a word of caution is in order. The ability to coprecipitate two proteins from a cellular extract is not proof that a particular interaction normally takes place in vivo. Additional experiments are needed to argue that a given interaction is not the result of mixing cell contents during extract preparation. Such evidence could include colocalization of the proteins or demonstration of functional relatedness.

When performing coprecipitation, it is important to precipitate from both directions (i.e., individually precipitating protein 1 and protein 2, and testing for the presence of protein 2 and protein 1, respectively). This is important in that it can provide further verification of an interaction between the proteins. It is also important because it is possible the interaction will only be detected in one direction. An inability to detect an interaction in one direction could be due to a variety of factors including obstruction of an interaction by the binding of

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the antibody or other affinity agent, or differences in pool size representation of each protein. For example, protein 1 may bind to many proteins besides protein 2, while most of protein 2 binds to protein 1. In this scenario, it would be anticipated that detection of their association will be most efficient when protein 2 is precipitated.

Critical Parameters and Troubleshooting

It is important to vary conditions of both the extract preparation and the coprecipitation to determine what is optimal. When starting from scratch, it is most prudent to use a range of lysis and precipitation conditions from less to more stringent in terms of the amount of salt and nonionic detergent. When no interaction is detected, it is worthwhile to use less stringent conditions (reduced salt with little or no nonionic detergent). In addition, it may be necessary to avoid any dilution of the whole-cell extract. This can be done by adding the protein A/G–Sepharose directly to the extract after an initial clarification centrifugation and by using smaller wash volumes. Depending on the strength and nature of the interaction, the precipitation can be done in the presence of a mixture of detergents that includes ionic detergents (for example, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS), similar to that described in RIPA buffer (*UNIT 3.8*).

In addition, it may be necessary to increase the expression levels of the proteins in question to be able to readily detect them by coprecipitation. A range of expression levels is recommended, because a level that is too high can lead to unregulated interactions (Feng et al., 1998). Alternatively, one can scale up the coprecipitation and use more than 0.5 to 1 mg of whole-cell extract (Feng et al., 1998). Here, the limiting factor is the concentration of the extracts, which must be high enough to allow the volume of the coprecipitation mixture to remain low. Larger-scale extract preparations may be necessary to generate more concentrated extracts. Finally, in cases of failure due to low abundance of the proteins in the host organism, one can overexpress a tagged version of one of the two proteins in the same or another host (such as *E. coli*), concentrate this protein by preimmobilization on an appropriate affinity matrix, and then incubate the affixed protein with extracts from the host organism.

The most important objective in these experiments is to generate as great a signal-to-noise ratio as possible and avoid problems of background. A variety of parameters can be changed to enhance the co-immunoprecipitation. Optimization of the precipitating antibody is one possibility. Protein A–Sepharose and protein G–Sepharose should give results comparable to anti-Ig serum. However, direct coupling of the antibody to Sepharose may lead to lower background and more quantitative precipitation. In addition, varying the ratio of antibody to whole-cell extract and the total amount of whole-cell extract is strongly suggested to determine the optimal amount of antibody that gives the most precipitation with the least amount of background. Affinity purification of the antibody may be necessary if the antibody immunoprecipitates additional cross-reacting proteins.

Additional approaches can be taken to minimize background. First, better clarification of the cell extract can be achieved by precentrifugation at 100,000 × *g*. These extracts can be directly used for coprecipitation without an intervening freezing step, which can increase the amount of protein precipitation. Second, both the lysis buffer and the coprecipitation buffer can be supplemented with 1% BSA to reduce nonspecific binding to the affinity matrix. Third, the whole-cell extract can be preincubated with protein A/G–Sepharose to remove nonspecific proteins that bind to the solid support. Fourth, the amount of salt and detergent can be increased in both the coprecipitation and the washes to reduce nonspecific binding. Fifth, increasing the number of washes may also help, although it may reduce the amount of specific protein that remains associated. Sixth, the expression levels of the proteins in question can be increased to generate a stronger signal that is above background binding. Alternatively, it may be possible to produce a whole-cell extract that is enriched for the proteins in question (e.g., by preparing a nuclear extract if the proteins are known to be in the nucleus). In instances where one of the proteins binds nonspecifically to Sepharose, the substitution of an agarose-based affinity matrix may help solve the problem. Finally, it may be necessary to generate a different set of reagents to precipitate the proteins in question (i.e., different antibodies and/or protein tag).

Anticipated Results

Provided suitable antibodies are available to the proteins in question and the physical interaction is stable under the coprecipitation conditions, it should be possible to detect an interaction between two proteins.

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Time Considerations

Once the extracts are prepared, coprecipitation can be done within 3 to 4 hr, yielding samples ready to load on a gel for SDS-PAGE and immunoblot analysis.

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Phizicky and Fields, 1995. See above.

General discussion of methodologies for detecting protein-protein interactions as well as their merits and drawbacks.

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QUANTITATION OF PROTEIN INTERACTIONS

20

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CHAPTER 20
Quantitation of Protein Interactions

INTRODUCTION

The highest level of protein structure is quaternary structure, formed by the noncovalent association of protein subunits. Usually the associating protein subunits have a folded, or tertiary, structure, although there are cases where unfolded proteins with only secondary structure directly associate to form folded multisubunit complexes (e.g., trimeric glucagon). Protein complexes can range from simple symmetrical homodimers formed of identical subunits to highly complex multisubunit structures such as viral capsids.

Protein-protein interactions are important, indeed vital, to many biological events and processes. Analysis of interactions between proteins and detailed analysis of molecular interfaces—generally done in vitro using isolated proteins—are the bases for understanding many biological processes. A number of biochemical and biophysical techniques can be applied to study specific interactions involving proteins. This information, together with high-resolution structural information obtained by X-ray crystallography and NMR (Chapter 17), can provide an understanding of the molecular basis of these interactions.

Methodologies for studying the identification and quantitation of protein interactions have become so important that the editors of this volume have decided to devote two chapters to these techniques. In Chapter 19, the emphasis is on the detection of specific protein interactions; in this chapter the material is more concerned with quantitative aspects—e.g., the measurement of binding strengths and rates of binding. Quantitation of binding is usually performed using biophysical techniques such as gel filtration, equilibrium dialysis, large-zone analytical size-exclusion chromatography (SEC), and titration microcalorimetry, to name a few; these techniques are reviewed in *UNIT 20.1*. Some of these methods rely on sophisticated equipment such as the analytical ultracentrifuge. It is recognized that many laboratories will not own such instruments, so the first units covering each method will be extended overviews designed both to supplement the information in the general chapter overview and to provide investigators with enough background to both follow the published literature and, if necessary, intelligently seek collaboration. These overviews will be complemented by actual experimental protocols to be published in future supplements.

In this vein, then, comes *UNIT 20.2*, the first extended overview. This unit discusses the measurement of protein interactions by optical biosensors (including the popular Biacore instrumentation). It includes a Strategic Planning section on how to proceed with an experiment—depending on whether the binding will be steady-state, kinetic, or competitive—and information on sample preparation, analysis of results, and troubleshooting of suboptimal experimental results. Other extended overviews currently in preparation will cover titration microcalorimetry, analytical centrifugation (equilibrium and sedimentation-velocity approaches), and size-exclusion chromatography, among others.

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Overview of the Quantitation of Protein Interactions

The biological function of many (and perhaps most) proteins involves reversible interactions with other proteins, nucleic acids, or other nonprotein ligands. Such interactions play many different roles in a wide range of cellular processes. A few examples are: (1) storing or transporting key metabolites (e.g., O_2 storage by myoglobin); (2) forming and maintaining the quaternary structure of multisubunit enzymes; (3) specific binding and recognition events (antigen-antibody, hormone-receptor, transcription factor–promoter); and (4) self-assembly of large structures (e.g., microtubules or chromatin). Thus the quantitative characterization of such interactions represents an important part of understanding the function of such proteins and their role in these cellular events.

The two fundamental properties that characterize any binding interaction are the stoichiometry (how many moles of each reactant combine to form the product) and the binding strength. The binding strength or "affinity" of the interaction is usually quantitatively expressed through either the equilibrium association constant, K_a, or its inverse, the dissociation constant, K_d, which for a reaction nA + mB $\leftrightarrow$ A_nB_m are defined by:

$$K_a = \frac{[A_nB_m]}{[A]^n[B]^m}$$

$$K_d = \frac{[A]^n[B]^m}{[A_nB_m]}$$

The choice of using K_a or K_d is largely a matter of personal preference. The concentration terms in Equation 20.1.1 may be expressed in any concentration units, but molar units are generally preferred. The binding strength can also be characterized by the change in Gibbs free energy for the reaction, ΔG, which is related to the equilibrium constant through the relation $\Delta G = RT\ln(K_d) = -RT\ln(K_a)$, where R is the gas constant and T is the absolute temperature. It should always be remembered that such energy changes are not absolute, but are always relative to some reference state, which conventionally is taken as a 1 M solution of each reactant (and hence the preference for equilibrium constants in molar units).

The various experimental methods differ considerably in their ability to determine the stoichiometry and/or K_d as well as in the range of values they can measure. Table 20.1.1 lists the techniques that will be covered in this unit and summarizes the type of information each gives and the approximate range of K_d values it can measure. The good news is that there are often several of these methods that can be appropriately applied to a given system. Moreover, the continued emergence of new and improved instruments, software, and protocols broadens the choices and reduces the time, effort, and/or amount of material required for obtaining the desired information.

SOME COMMENTS ON TECHNIQUES

Most of the methods in this unit detect binding interactions through a change in mass as binding occurs. In addition to mass spectrometry itself, this group includes sedimentation equilibrium (analytical and micropreparative), surface plasmon resonance (SPR), size-exclusion chromatography (SEC) with on-line light scattering, and chemical cross-linking. Techniques such as large-zone analytical SEC, sedimentation velocity, and some of the electrophoresis methods, while not strictly mass-based since the changes in hydrodynamic properties or mobility are related to changes in molecular shape as well as mass, can nonetheless reasonably be grouped with the true mass techniques. Changes in spectroscopic properties such as fluorescence polarization (Heyduk et al., 1996) may also be related to mass changes and hence certain spectroscopic approaches also belong in this group.

As might be expected, methods that are members of this group are usually capable of determining stoichiometry, but some of these methods are not necessarily able to measure the equilibrium constant. For example, mass spectrometry, cross-linking, and light scattering/SEC are generally excellent at determining stoichiometry, but the amount of the higher-mass complex that they detect may not accurately reflect its proportion at equilibrium. It is also important to remember that some methods, such as sedimentation velocity and light scattering/SEC, involve dynamic physical separa-

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Table 20.1.1 Summary of Characteristics for Some Important Methods for Characterizing Protein Interactions

Technique[a]	Basis for detection	Binding properties measured (and range of K_d)	Minimum sample mass or volume needed[b]	Typical analysis time[c]	References	Comments
Mass spectrometry	Change in molecular mass	Stoichiometry	0.001 µg; <1 µl	15 min	Loo, 1997; Roepstorff, 1997; Farmer and Caprioli, 1998	May not detect weaker interactions; very helpful prelude to centrifugation methods, especially for glycoproteins
Chemical cross-linking	Change in molecular mass	Stoichiometry	5 µl[d]	30 min + readout	Loster and Josic, 1997; Kunkel et al., 1981	Useful and fairly simple approach to determining quaternary structure and stoichiometry
SEC with light scattering	Change in solution mass	Stoichiometry	100 µg (20 kDa); 10 µg (200 kDa); 5 µl	30 to 60 min	Harding et al., 1992; Wen et al., 1996	Gives true mass, independent of molecular shape
Equilibrium dialysis and gel permeation, ultrafiltration, gel chromatography (Hummel-Dreyer)	Partitioning of free and bound ligand	Stoichiometry (K_d ~10^{-1} to 10^{-13} M)	10 µl[d]	15 min to 24 hr + readout	Andreu, 1985; Kido et al., 1985; Sophianopoulos and Sophianopoulos, 1985; Sebille et al., 1990	"Low tech" and often labor-intensive, but still very useful and adaptable
Large-zone analytical SEC	Change in hydrodynamic size	Stoichiometry (K_d ~10^{-4} to 10^{-13} M)	1000 µl[d]	30 to 60 min + readout	Ackers, 1973; Valdes and Ackers, 1979; Nenortas and Beckett, 1994	Wide range of potential on-line or off-line detection methods; may be labor-intensive depending on degree of automation
Micropreparative analytical ultracentrifugation (sedimentation equilibrium)	Change in solution mass	Stoichiometry (K_d ~10^{-3} to 10^{-13} M)	100 µl[d]	12 to 24 hr + readout (4 to 6 samples per rotor)	Darawshe et al., 1993, 1995	Requires only preparative centrifuges; fewer data points and more labor-intensive than on-line instruments, but much greater specificity and sensitivity through off-line detection

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Spectroscopic methods	Change in spectroscopic property	Molar ratio stoichiometry (possibly); conformational changes (K_d ~10^{-5} to 10^{-11} M)	—[e]	0.2 to 2 hr	Oberfelder and Lee, 1985; Heyduk et al., 1996; van Holde et al., 1998	Applicability depends on nature of protein and/or ligand; chromophore label may be required
Titration microcalorimetry	Heat release or uptake	Molar ratio; ΔH; Δc_p (K_d ~10^{-3} to 10^{-9} M)	100 µg; 2000 µl	3 hr	Bundle and Sigurskjold, 1994; Fisher and Singh, 1995; Doyle, 1997	Universal signal; works well for small molecules; useful additional thermodynamic information
Sedimentation equilibrium (in analytical ultracentrifuge)	Change in solution mass	Stoichiometry (K_d ~10^{-3} to 10^{-9} M)	10 µg; 100 µl	12 to 24 hr per rotor speed (9 to 21 samples)	Rivas and Minton, 1993; Laue, 1995; Schuster and Toedt, 1996	Powerful method when there is a significant change in mass; much more accessible with modern hardware and software
Sedimentation velocity (in analytical ultracentrifuge)	Change in sedimentation coefficient	Stoichiometry; conformational changes (K_d ~10^{-3} to 10^{-9} M)	10 µg; 400 µl	2 to 5 hr (3 to 7 samples)	Schuster and Toedt, 1996; Lobert et al., 1997; Stafford, 1997	Newer software makes data analysis fast and user-friendly; generally not as good as sedimentation equilibrium for K_d
Surface plasmon resonance	Change in mass bound to surface	Stoichiometry; kinetics (K_d ~10^{-3}-10^{-13} M)	0.01 µg; 100 µl	15 to 30 min (1 analyte, up to 4 surfaces)	Myszka, 1997; Schuck, 1997; Morton and Myszka, 1998	Broad range of K_d; high throughput; fairly insensitive to contaminants; potential problems from surface coupling

[a]Techniques that primarily give information about stoichiometry are listed first, followed by those with good quantitation of binding affinity, with the latter arranged roughly in order of cost and complexity of the instrumentation.

[b]Approximate minimum sample mass (detection limit) and minimum sample volume. For weaker interactions the sample requirement is often dictated more by the volume requirement, since it is necessary to have this volume of solution at a concentration high enough to saturate the interaction. These figures will vary by an order of magnitude or more depending on the equipment and exact protocol employed.

[c]This is the approximate time needed to carry out the experiment. For methods in which more than one sample may be run simultaneously, the number of samples is noted in parentheses. For methods in which the readout of concentration is performed separately (e.g., by ELISA or scintillation counting) the time for that step is not included.

[d]Required mass depends on detection method; for radiolabeling, ELISA, or fluorescence detection the mass required may be extremely small.

[e]Required mass and volume depend on detection method.

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tion of components and are not true equilibrium methods. With these methods, the results may depend on the *rates* of association-dissociation as well as the equilibrium constant. For relatively weak interactions with relatively fast kinetics, the band or boundary that is seen is a so-called "reaction boundary" and does not necessarily correspond to any single species or any particular stoichiometric complex. This complication does not apply to sedimentation equilibrium, even though there is some separation of components, because association-dissociation equilibrium is maintained at every position in the centrifuge cell. Large-zone analytical SEC also can be regarded as a true equilibrium technique because the concentration and composition of the plateau region matches what was present before the sample was loaded onto the column.

One common drawback of these methods based on mass is that they may be insensitive to the binding of peptides or other ligands having a mass that is small compared to that of the protein. Under certain circumstances it may therefore be desirable to deliberately increase the mass of such ligands. For example, a peptide might be fused with a larger polypeptide or a small ligand modified by adding a defined polyethylene glycol polymer. The caveat here is, of course, that such modifications must not interfere with binding, so this approach usually requires knowledge that some region of the ligand is *not* important for binding.

Surface Plasmon Resonance

SPR-based sensors have several unique characteristics relative to the other mass-based methods, which warrant some further discussion. First, SPR is unique among these methods in that the kinetics of the association and dissociation events can be measured explicitly, and this kinetic information can provide insights that may be difficult to obtain from equilibrium information alone. SPR actually provides two fairly different approaches to characterizing the binding affinity. In one approach, the equilibrium association constant is determined from the kinetic data by first determining the association and dissociation rates and then calculating their ratio. The alternative approach involves exposing the reactant solution to the measurement surface for a long time to allow the binding to reach equilibrium. The magnitude of this binding signal is then analyzed as a function of reactant concentration to obtain the equilibrium constant, in a fashion similar to other equilibrium methods.

Another significant difference in SPR is that one of the reactants is coupled to a surface. In principle, this should not matter, provided that this coupling does not produce steric hindrance or involve chemically modifying residues important for the interaction being studied. Random coupling methods, however, have the potential for producing a range of orientations and binding properties, so coupling via a specific site and/or in a specific orientation is preferred for detailed quantitative studies. Further, surface binding can be fundamentally different than solution binding if there is the potential for more than one molecule on the surface to simultaneously interact with a single analyte molecule in solution. A failure to properly consider and treat multivalent interactions on the surface can, and has, led to errors of many orders of magnitude in binding constants obtained by SPR (Kalinin et al., 1995; Ladbury et al., 1995). Thus independent knowledge of the reaction stoichiometry is quite helpful for the design and interpretation of SPR experiments. With current procedures (Schuck, 1997; Morton and Myszka, 1998; *UNIT 20.2*) such problems arising from multivalent interactions, as well a number of other potential artifacts in SPR, can usually be avoided.

SPR is generally an excellent tool for studying protein-ligand interactions and interactions between two different proteins, but the required surface coupling limits its utility for characterizing protein self-associations. Lastly, it should not go unmentioned that one of the great virtues of SPR methods is that the amount of material required is usually quite small, particularly for the component bound to the measurement surface.

Analytical Ultracentrifugation (Sedimentation Equilibrium and Sedimentation Velocity)

Once relegated to a few dedicated specialists, with improved hardware and automation this technique is again becoming widely used and more accessible to nonspecialists, and the quantity of sample required is also reduced. Among the strengths of analytical ultracentrifugation are that the governing principles are simple and well understood, and the theory and practice has been developed over many years.

Sedimentation equilibrium has many virtues (and a long history of success) for detailed studies of complex interactions, among which are: (1) it is a true solution equilibrium method; (2) it can cover a broad range of concentrations (~5 μg/ml to >100 mg/ml for

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proteins); (3) there are few restrictions on the choice of solvents; and (4) it can be applied over a fairly wide temperature range (0° to 40°C for the Beckman XL-I). Its primary drawback is that reaching equilibrium typically requires 12 to 24 hr for a 3-mm solution column, so the throughput is fairly low (but 9 to 21 samples can be run simultaneously), and some proteins are not sufficiently stable over these long time periods. The use of even shorter solution columns (<1 mm) with interference optics reduces equilibration time to a few hours or less, but results in fewer data points and less overall accuracy. This short-column approach is excellent for determining stoichiometry, for survey experiments, and for studying changes in weight-average mass as a function of ligand concentration or mixing ratios of different proteins.

It should not be overlooked that sedimentation equilibrium experiments can be performed using preparative centrifuges, and this approach has advantages over the dedicated analytical instruments (Rivas and Minton, 1993). The collection of fractions at the end of the experiment allows any off-line method to be used to follow the concentration distribution, greatly extending the range of concentrations and specificity of detection methods. This also permits further separation of individual components by electrophoresis or chromatography prior to detection.

Sedimentation velocity experiments require only 2 to 5 hr, and thus can be employed for labile proteins, and are now often done at protein concentrations of 10 to 100 μg/ml instead of the 2 to 10 mg/ml common in the past. Stafford's *dc/dt* method of data analysis (Stafford, 1994) has been a boon to the field, and the stoichiometry of tight complexes can often be determined simply by observing the appearance of new peaks as reactants are mixed in various proportions (Stafford, 1997). Other recent developments in data analysis (Philo, 1994; Stafford, 1997; Schuck, 1998) now allow the simultaneous determination of diffusion coefficients along with the sedimentation coefficients, and thus calculation of the true mass, for nonassociating monomers as well as tightly associated oligomers or complexes, provided there is little dissociation during the experiment. In general, though, individual species or complexes are not resolved, and the data are analyzed to give an overall weight-average sedimentation coefficient that changes systematically as binding or self-association occurs. This latter approach is particularly useful for studying ligand binding when that binding is linked to protein self-association, as exemplified by recent work on drug binding to tubulin by Lobert and Correia (Lobert et al., 1998). The fitting of weight-average sedimentation data to obtain K_d can be complicated by the fact that the sedimentation coefficients depend on shape and conformation as well as mass, and thus it is generally not possible to accurately predict the sedimentation coefficients of oligomers or protein-protein complexes, and these must be treated as additional adjustable fitting parameters. On the other hand, the sensitivity to conformation can allow studying the binding of even very small ligands when that binding is linked to a conformational change. The latest development in using sedimentation velocity for studying interactions is use of finite-element numerical methods to directly simulate and fit a binding model (Schuck, 1998; Stafford, 1999). This approach can potentially obtain information about kinetics as well as K_d.

Titration Microcalorimetry

In titration microcalorimetry, interactions are detected through the heat that is released or absorbed as a binding event occurs. The fact that this heat of reaction signal is so universal, and does not require a significant change of mass or the presence of any chromophores, is one of the great strengths of this method. It is important to note that this method does not measure the true binding *stoichiometry* but rather measures only the *molar ratio* at which the reactants combine—i.e., it cannot distinguish a 1:1 from a 2:2 stoichiometry. This method can be applied to self-associating systems by detecting dissociation as the protein is diluted into buffer, but the limited concentration range, and lack of information about true stoichiometry, limit its utility in this area.

Titration calorimetry is often the method of choice for binding of low-molecular-weight ligands, particularly when the affinity is fairly low. Moreover, it is unique in directly giving information about the binding enthalpy, ΔH, in addition to measuring equilibrium constants and molar ratios. Another unique aspect of this technique is that the proton binding or release associated with a protein-protein or protein-ligand interaction can be quantitated simply by changing buffer systems (Doyle et al., 1995; Baker and Murphy, 1996).

On the other hand, phenomena other than the binding event of interest can sometimes generate significant amounts of heat, so titration calorimetry also has a unique set of arti-

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facts that must be avoided. Among the potential problems are heat generated by small differences in pH or salt concentration between the titrant and the solution being titrated, and aggregation and precipitation of proteins induced by the stirring of the calorimeter cell. The heat due to solvent differences can of course be eliminated by thorough dialysis, but when studying binding of low-molecular-weight compounds, dialysis is often not possible. Limited solubility of the reactants can also create difficulties and force the use of large injection volumes, which generally compromises the signal/noise. Overall, though, this technique can be very useful, can give highly precise data, and the instrumentation and ease of use continues to improve.

Methods Based on Separation or Partitioning

The glamour and popularity of the some of the "high-tech," instrumentation-intensive methods should not overshadow a number of relatively simple and inexpensive approaches for studying protein interactions. For protein-ligand interactions, equilibrium dialysis or ultrafiltration often work well, and are still primary methods for studying drug binding to serum proteins (Sophianopoulos and Sophianopoulos, 1985; Sebille, 1990; Sebille et al., 1990). Gel filtration media can be used in either batch mode (Kido et al., 1985), for small-zone chromatographic analysis (Hummel-Dreyer analysis and its variants; Hummel and Dreyer, 1962; Andreu, 1985), or for large-zone (frontal) chromatography (Valdes and Ackers, 1979; Nenortas and Beckett, 1994). Virtually any method with good precision can be used to monitor the concentration of the protein or ligand following separation (or on-line monitoring may be used for chromatography), allowing great flexibility and an enormous concentration range. A common problem with these approaches is that the ligand or protein may bind to the membranes or resins. Furthermore, since transport is involved, one must be cautious about whether the measurements reflect equilibrium rather than kinetic properties. The material requirements may also be high unless some effort is devoted to keeping volumes small, and these approaches can be fairly labor intensive. Additional separation-based methods for protein-ligand interactions that have been used successfully are steady-state and counterion electrophoresis and isoelectric focusing (Oberfelder and Lee, 1985) and affinity-chromatography approaches (Winzor and De, 1989).

THE IMPORTANCE OF DATA ANALYSIS METHODS

Another common theme among these techniques is the central role of numerical methods for analysis and interpretation of the data, and this aspect has proven daunting for some researchers. Fortunately, today's desktop computers allow very sophisticated analyses to be done quickly and routinely, and the software has become increasingly user-friendly. Equally important, the data now are often acquired directly in digital form, and on-line computerized instrument control permits unattended operations and more complex data-acquisition protocols.

To see the tremendous impact the sum of all these phenomenon can have, one need look no further than at analytical ultracentrifugation. With new computerized instrumentation, formerly onerous data acquisition and analysis chores now take only seconds, and new numerical methods for analysis have added new capabilities and approaches. The net result is that a field that had all but disappeared has been rejuvenated.

In the area of data analysis, one frequently encounters the need to formulate specific models for use in analyzing the data. For example, protein self-association studies by large-zone SEC or sedimentation equilibrium are typically fitted to a specific assembly pathway such as monomer $\leftrightarrow$ dimer $\leftrightarrow$ tetramer. In such situations, it is always important to critically analyze whether the right model has been chosen, and to try to get independent information to support that choice of model. In this regard, information about the reaction stoichiometry (or at least the limiting assembly states) is often especially valuable.

Another important phenomenon in data analysis is the more widespread use of "global fitting" techniques. In global fitting, multiple experiments taken under different conditions (and possibly by different techniques) are analyzed simultaneously, using a common set of parameters to describe the molecular interactions. Such global analysis provides a much more robust analysis, and often allows more information to be extracted from a given set of experiments. This approach is fairly commonly used for certain techniques (e.g., analytical ultracentrifugation and SPR data), but is still seldom used for data from more than one type of instrument.

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SENSITIVITY TO CONTAMINANTS AND PROTEIN QUALITY

In general, the acquisition of accurate quantitative data on protein interactions will require highly purified, fully active protein samples, but a few of these techniques can be quite insensitive to the presence of contaminants or other proteins present as carriers. This is an area where the SPR sensors particularly excel, and titration calorimetry is also fairly insensitive to contaminants or carrier proteins.

Unfortunately, it is not uncommon for even highly purified protein samples to contain some inactive or partially active material, and further degradation may occur during the course of the measurements. This will usually limit the accuracy and reproducibility of the quantitative results, and possibly may even lead to false interpretations of the data. For example, proteins normally found as tightly associated oligomers often contain a small fraction of partially-denatured monomers that do not self-associate, or at least do so very weakly. Such species are often termed "incompetent monomers" in the analytical ultracentrifugation community. Suppose one is trying to quantitate the reversible dissociation of this oligomer at low concentrations in order to establish whether the oligomer exists at physiologically relevant concentrations. The presence of any incompetent monomer can result in a large increase in the *apparent* K_d for the dissociation when little reversible dissociation occurs over the experimentally accessible concentration range. Thus, extrapolation of such data to a low physiological concentration could lead to a false conclusion about the physiologically relevant quaternary structure.

Similarly, titration calorimetry is sometimes employed to study interactions with dissociation constants in the nanomolar range under conditions where the reactant concentrations are two to three orders of magnitude higher than the K_d. Under such conditions, it may be very difficult to distinguish a sample in which the majority of the protein binds extremely tightly (outside the range of the instrument) but a small portion of the protein binds more weakly, from one where 100% of the protein has a K_d that is close to the measurement limit of the instrument.

Such potential artifacts from inactive or partially active species can, of course, be detected through inconsistencies in the apparent K_d values for samples studied at different concentrations. That is precisely why it is often important to incorporate data from multiple experiments into the data analysis, and to cover as wide a portion of the binding isotherm as possible.

Overall, it is always advantageous to have highly pure, well-characterized, and stable protein samples. Before embarking on a detailed analysis of a complex set of protein interactions, it may be wise to devote some further effort to purification and to finding solvent conditions that promote stabilization. For proteins with poor stability, it will also be prudent to choose methods that are more rapid, and perhaps to work at lower temperatures.

THE ADVENT OF RECOMBINANT PROTEINS ENHANCES OUR CAPABILITIES FOR STUDYING PROTEIN INTERACTIONS

Biotechnology has had a profound influence on many areas of science and medicine, including the study of protein interactions. Many of the proteins being studied today are produced by recombinant technology, and would otherwise be unavailable in sufficient quantity (or totally unknown if they have been found through genomic or proteomic techniques). The quality of recombinant proteins (purity and homogeneity) can also often be superior to those from natural sources, in part because starting purification with larger amounts of protein allows more extensive purification protocols and more rigorous exclusion of side fractions.

Aside from issues of quality and quantity, a major virtue of recombinant proteins is the ability to engineer them in ways helpful for interaction studies. This is particularly useful for adding new, specific chemical groups such as cysteines for purposes of specifically immobilizing (e.g., SPR) or tagging the protein (e.g., fluorophores or isotopes). Such engineering may also involve adding new sequence motifs such as epitopes for commercially available antibodies or even entire functional domains such as green fluorescent protein.

Protein engineering can also be useful in enhancing certain physical properties of the protein. Among these are improving stability or solubility, removal of sites leading to heterogeneity (N-terminal processing, glycosylation), or even removing flexible loops. While some of these aspects may be more of interest in obtaining proteins better suited for high-resolution structural determination, lowering the chemical and structural heterogeneity may also be desirable before undertaking extensive interaction analysis of complex systems.

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Last, but certainly not least, interaction studies done with site-directed mutants and/or proteins with deletions of individual functional domains allow a much more detailed interpretation of the interaction data and strongly enhance the analysis of structure/function relations.

THE VIRTUES OF USING MULTIPLE TECHNIQUES

Seldom can any one method give complete and fully reliable information about protein interactions. Each method has certain strengths and weaknesses, and may depend on certain models and assumptions. Therefore it is highly desirable, and sometimes necessary, to use multiple techniques in studying any protein interaction. Fortunately many of the techniques covered in this unit are highly complementary to one another, and this is definitely a field where the whole is greater than the sum of its parts.

It is always important to characterize the state of association of the protein(s) being studied. Such studies of self-association may be either the desired endpoint of an interaction analysis, or only preliminary to studying a protein-ligand interaction or the interactions between different proteins. Mass spectrometry provides, at the very least, an excellent beginning and foundation for other methods of studying self-association by verifying the covalent structure and determining an accurate monomer mass, and may also provide information about the presence of higher oligomers or binding of metal ions or other ligands (Anderegg et al., 1997).

Light scattering/SEC is also very useful (and very quick) for determining whether the predominant form is a monomer, dimer, or other oligomer. While this is not usually a good method for directly measuring the equilibrium constants for self-associating systems, it can be very helpful in identifying limiting stoichiometries, and may identify other assembly intermediates or the presence of incompetent monomer. Light scattering/SEC therefore can be an excellent prelude or adjunct to sedimentation equilibrium or large-zone analytical SEC (often the best methods for fully characterizing a self-associating system) by providing independent information about appropriate assembly models. In principle the combination of on-line scattering with large-zone SEC should make an excellent tool for measuring binding affinities of mixed- or self-associations, but this has not yet been extensively exploited, and the sensitivity of the scattering detectors may not be sufficient for studying stronger interactions ($K_d < 10^{-7}$ to 10^{-8} M).

A wide variety of methods can be applied to interactions between different proteins or protein-DNA interactions (so-called "mixed" or "heterogeneous" associations), and many are complementary rather than redundant. Once again a knowledge of stoichiometry can be very helpful in designing experiments and selecting proper models for data analysis, and mass spectrometry, light scattering/SEC, and chemical cross-linking can be very helpful in this regard.

In many cases, several techniques may be appropriate for determining the K_d, and if so it is certainly helpful to use more than one, particularly for complex associations or when the information is critical. One field where there has been a particularly nice synergy between methods such as sedimentation equilibrium, SPR, light scattering/SEC, and titration calorimetry has been in studies of interactions between protein hormones and their receptors (Ward et al., 1994; Zhang et al., 1997; Philo and Hensley, 1998). By confining receptors to the surface of SPR sensors it has also been possible to more closely mimic the situation when the receptor is embedded in the cell membrane, and even multisubunit receptor complexes have been assembled on the sensor surface (Kalinin et al., 1995; Myszka et al., 1996). Comparison of such data with those from solution methods such as sedimentation equilibrium or titration calorimetry may allow a more thorough understanding of the importance of having the hormone interact simultaneously with multiple receptor subunits, and determination of the energetic consequences of having the receptors preoriented in a fashion enabling such interactions.

SOME THOUGHTS ON CHOOSING METHODS

As mentioned earlier, the fact that most of the methods are based on changes in mass often limits their application to studying binding of low-molecular-weight compounds, so for such materials, titration calorimetry or a "low-tech" approach like equilibrium dialysis may be particularly advantageous. For protein-protein or protein-DNA interaction studies, the choice of method(s) for determining the K_d will often be dictated by the magnitude of that K_d. Both titration calorimetry and sedimentation equilibrium lack the sensitivity to measure K_d values below ~10^{-8} to 10^{-9} M. Stronger interactions with K_d values down to ~10^{-12} M can be studied by SPR or fluorescence techniques. By

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coupling them to highly sensitive radiometric, fluorometric, or ELISA assays, techniques such as equilibrium dialysis, ultrafiltration, micropreparative analytical ultracentrifugation, or large-zone analytical SEC can also study K_d values in the picomolar range or lower. At the other extreme, weak interactions with K_d values up to ~10^{-3} M can be measured by analytical ultracentrifugation, titration calorimetry, SPR, equilibrium dialysis, or ultrafiltration. With very weak interactions, reactant solubility may limit the application of titration calorimetry, while nonspecific binding to the surfaces can be problematic for SPR, dialysis, and ultrafiltration.

In other cases the choice of technique to apply to obtain the K_d of a mixed or self-association may be dictated by the amount of material that is available. For the less sensitive techniques such as sedimentation equilibrium and titration calorimetry, where this is most likely to be an issue, as a rule of thumb the minimum amount of protein needed to run one sample with good signal/noise ratio is ~10 µg for sedimentation equilibrium (with a 3-mm column), and ~100 µg for titration calorimetry. However, the amount of material needed is often dictated not by the inherent detection limits of a particular instrument, but rather by the necessity to run at least one sample at a high enough concentration to promote essentially full association (i.e., 10 times the K_d or preferably higher). Thus, the material requirement is often proportional to the product of the K_d and the minimum volume needed for this high-concentration sample. For the more sensitive methods, this volume requirement may actually be dictated more by sample-handling considerations than by how much material actually enters the instrument. For sedimentation equilibrium, the minimum volume is ~100 µl, for sedimentation velocity ~400 µl, and for titration calorimetry ~2 ml. In SPR the material requirements for the reactant that will be coupled to the sensor surface is quite small; material requirements are usually only a potential concern for the reactant in solution. For flow-based SPR sensors the minimum volume for the solution reactant depends on flow rate and injection length, which in turn vary with the kinetics of the process and whether the instrument is operated in a kinetic or equilibrium-binding mode, but this volume is typically ~100 µl. In large-zone SEC, the volume required depends on the column volume but is often several milliliters (or more).

OBTAINING ADDITIONAL THERMODYNAMIC INFORMATION

Additional insight into protein interactions can be obtained by extracting solution thermodynamic quantities from the experimental data. As mentioned previously, titration calorimetry measures the binding enthalpy, ΔH, directly (and this quantity can be measured easily even when the K_d is too low to be measured). When the K_d is also known, the entropy change can then be calculated from the relation $\Delta G = RT\ln(K_d) = \Delta H - T\Delta S$. The magnitudes of ΔH and ΔS may provide some insight into the nature of the interaction (e.g., whether it is predominantly hydrophobic or electrostatic). However these net quantities are sums of contributions from many effects, and are often dominated by interactions with the solvent, so structural interpretation is risky at best.

A more promising approach to structural interpretation of the thermodynamic data comes from examining the change in heat capacity, Δc_p. This can be obtained by measuring the calorimetric ΔH at several temperatures and taking the slope of a plot of ΔH versus T, through the relation $\Delta c_p = \delta(\Delta H)/\delta T$. This Δc_p can then be interpreted in structural terms, as either a change in buried surface area when the complex is formed (Murphy and Freire, 1992) or as the number of amino acid residues that go from a disordered to an ordered state as a consequence of the interaction (Spolar and Record, 1994).

The apparent, or van't Hoff, enthalpy change, ΔH_{vH}, can also be obtained by measuring the K_d as a function of temperature (using any method) if it is assumed that ΔH and ΔS are constant with temperature (at least over some narrow range). This is done by plotting $\ln(K_d)$ versus $1/T$ (a van't Hoff plot), taking the slope of a line through the data, and using the relation $\Delta H_{vH} = R\delta(1nK_d)/\delta(1/T)$. The calorimetric and van't Hoff enthalpies may differ significantly. The calorimetric ΔH includes *all* heats associated with the overall biochemical reaction, whereas ΔH_{vH} includes only those reactions that directly influence the K_d, and thus may not include heats associated with solvation, ionization, or conformational changes if those processes have no net effect on the binding free energy (Bundle and Sigurskjold, 1994).

It is also possible to take advantage of the temperature dependence of equilibrium constants to extend the range of affinities that can be covered by any given method. Since most reactions are exothermic (negative ΔH), they

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become weaker as the temperature is increased. Thus for studying very strong affinities it may be helpful to determine the K_d at high temperature and then correct this value to lower temperature using measured values for ΔH (and preferably Δc_p also). This approach has been used, for example, to study high-affinity antigen-antibody binding (Schwarz et al., 1995).

THERMODYNAMIC LINKAGE RELATIONSHIPS

The concept of thermodynamic linkage has provided a powerful tool for probing and understanding protein interactions, and also serves as a basis for extending the range of affinities that can be probed. Consider first a protein, P, that can bind a ligand, L, and that also self-associates to form dimer. The ligand binding and association reactions form the thermodynamic cycle, illustrated in Figure 20.1.1. Because the total change in free energy must be independent of the pathway, if the dimer binds ligand with a different affinity than the monomer, it must also be true that the liganded form dimerizes differently than the unliganded form, such that $\Delta G_L - \Delta G_L{}^d = \Delta G_d{}^L - \Delta G_d$ (or equivalently $K_L/K_L{}^d = K_d{}^L/K_d$).

How does this exercise help us? Suppose the dimer binds ligand more strongly than monomer, so strongly that we cannot measure the affinity. This linkage relationship says that if we can determine the (lower) affinity of the monomer, we need only measure the monomer-dimer dissociation equilibrium of the unliganded and liganded forms, and we may then calculate the ligand affinity of the dimer. This very simple but powerful idea has been exploited with tremendous success, for example, by Gary Ackers and his co-workers in detailed studies of oxygen binding and subunit assembly for hemoglobin (Turner et al., 1981; Ackers, 1998), in probing protein-DNA interactions (Koblan and Ackers, 1991), and in many other systems.

These same linkage concepts can also be applied when the protein's affinity for a ligand varies with pH, ions such as calcium, or effectors such as nucleotide phosphates, rather than through self-assembly. For example, since titration calorimetry can fairly easily quantitate proton uptake and release, a reaction whose affinity is too high to be studied at neutral pH can be studied at a pH where the binding is weaker, and then corrected to neutral pH conditions using the linkage relations and the difference in proton binding (Doyle et al., 1995; Baker and Murphy, 1996).

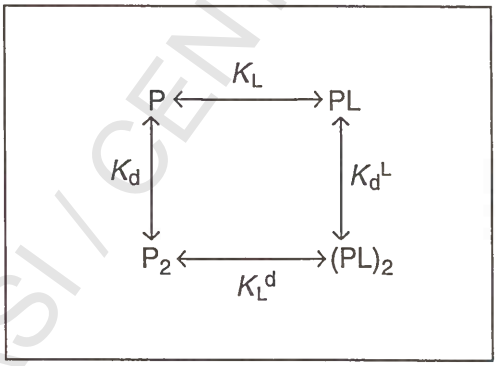

Figure 20.1.1 Thermodynamic cycle for ligand binding and association reactions. K_L and $K_L{}^d$ are the association constants for ligand binding for the monomer and dimer, respectively, and K_d and $K_d{}^L$ are the monomer-dimer association constants for the unliganded and liganded form of the protein, with corresponding free energies ΔG_L, $\Delta G_L{}^d$, ΔG_d, and $\Delta G_d{}^L$.

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Good introductory text; strong on spectroscopy and centrifugation methods

Johnson, M.L. and Frasier, S.G. 1985. Nonlinear least-squares analysis. *Methods Enzymol.* 117:301-342.

Good overview of the fitting of experimental data (a key part of all protein interaction studies), with some examples for ligand binding.

van Holde et al., 1998. See above.

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Winsor, D.J. and Sawyer, W.H. 1995. Quantitative characterization of ligand binding. John Wiley & Sons, New York.

An excellent but heavily mathematical text covering theory, data analysis, and interpretation. Methods based on phase separation, spectral changes, affinity and gel chromatography, and competitive binding are discussed in some detail, with some coverage of sedimentation, calorimetry, and other techniques. An entire chapter is devoted to DNA-ligand interactions (also see APPENDIX 5A).

INTERNET RESOURCES

rasmb-manager@bbri.org (subscriptions)

rasmb@rasmb.bbri.org (to post questions)

Reversible Associations in Structural and Molecular Biology (RASMB) e-mail list server. This e-mail group list is for discussions and questions regarding the study of reversible interactions. The dominant focus is analytical ultracentrifugation (and nearly all the experts participate), but tremendous expertise in many other relevant areas is represented as well.

http://www.bbri.org/RASMB/rasmb.html

Reversible Associations in Structural and Molecular Biology (RASMB) Web site. Distributes public domain software for analytical ultracentrifugation.

http://www.cauma.uthscsa.edu

Distributes public domain software for analytical ultracentrifugation. Also maintains an archive of the RASMB discussions and tutorials on sedimentation methodology.

http://www.hci.utah.edu/groups/interaction/index.htm

This site by the Protein Interaction Facility at the Huntsman Cancer Institute nicely describes the basics of SPR, analytical ultracentrifugation, and titration calorimetry, and is the distribution point for the CLAMP package of public-domain SPR analysis software.

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http://www.beckmancoulter.com/beckman/biorsrch/prodinfo/xla/xlaprod.asp

Analytical ultracentrifugation products from Beckman Coulter. Useful application notes and bibliographies.

http://www.calscorp.com

Calorimetry Sciences Web site. Useful calorimetry application notes and bibliographies.

http://www.microcalorimetry.com

Microcal Web site. Useful calorimetry application notes and bibliographies.

http://www.panvera.com/ls/fpabout.html

Panvera Web site (fluorescence polarization). Useful application notes and bibliographies.

http://www.wyatt.com

Wyatt Technologies Web site (light scattering). Useful application notes and bibliographies.

http://www.biacore.com

BIAcore Web site (SPR). Useful application notes and bibliographies.

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Measuring Protein Interactions by Optical Biosensors

Optical evanescent wave biosensors, such as surface plasmon resonance, resonant mirror, or waveguide biosensors, have been increasingly used during the last several years for the characterization of protein-protein interactions (Schuck, 1997a; Margulies et al., 1993, 1996; van der Merwe and Barclay, 1996; Malmborg and Borrebaeck, 1995; Garland, 1996). This includes both the determination of equilibrium binding constants, which reveal the magnitude of the binding energy, as well as the determination of the bimolecular rate constants describing the kinetics of the interaction.

Studying protein interactions at a surface, as opposed to solution methods, has at least two fundamental advantages. First, the magnitude of the signal involved is independent of the affinity of the interaction over a wide range. Because one binding partner is confined to the surface (in the following referred to as ligand), the number of free binding sites that are under observation in an experiment is determined predominantly by the surface immobilization procedure (requiring usually only very small amounts of material). Therefore, the sensitivity of the method is limited essentially by the availability of the soluble binding partner (the analyte) in a well-defined state, at concentrations in the order of the inverse affinity constant. This allows studies of interactions with affinities spanning a range of at least 10^5 to 10^{10} M^{-1}, and makes this method very attractive, in particular, for high-affinity interactions. Second, the separation of the surface-immobilized sites from the mobile binding partners in solution, if combined with an appropriate fluidic handling system, is virtually ideally suited for the real-time observation of the kinetics of the binding processes. Chemical on-rate constants of up to 10^5/Msec to 10^6/Msec and off-rate constants of between 10^{-5}/sec and 10^{-1}/sec are generally accessible. Information on the chemical on-rate constants, and on the lifetime of the complexes formed, can be of great value, for example, in the study of interactions involved in cellular signaling processes.

However, these fundamental advantages are gained at the expense of additional experimental difficulties intrinsic to surface binding studies. These include the need for immobilization of one reactant at a relatively high local surface density (and the execution of binding studies under these conditions), the problems of nonspecific surface binding, possible multiple attachments to the surface through multivalent interactions, the problem of mass transport in kinetic experiments, and finally the possibility of surface potentials influencing the thermodynamics of the interaction (Leckband, 1997; Leckband et al., 1995).

Although the different commercial instruments for optical biosensing are based on slightly different physical principles, they all exploit surface-confined electromagnetic fields for the precise and real-time measurement of the refractive index of the medium in the immediate vicinity of the sensor surface (Garland, 1996; Knoll, 1998; Lukosz, 1991). When ligands are immobilized to the sensor surface, reversibly interacting analytes will accumulate at the sensor surface when brought into the solution above. By virtue of the difference of the refractive index increments of proteins and most aqueous buffer solutions, an increase of the total refractive index at the surface will then be reported (Fig. 20.2.1). Removal of the analyte from the solution causes dissociation of the reversibly bound molecules from the surface sites, which is accompanied by a decrease in the refractive index at the sensor surface, generating a decrease in the signal. Since the changes in signal are, to a good approximation, proportional to the changes in the mass of surface-bound analyte, the steady-state signals can be analyzed in terms of the law of

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mass action and Langmuir isotherms. The time course of the signal can be interpreted in the context of chemical binding kinetics. This basic principle, and consequently the derived strategies for studying protein-protein interactions, as well as most of the experimental procedures and potential complications, are the same in the different commercial instruments. Among the manufacturers are Biacore (*http://www.biacore.com*), Affinity Sensors (*http://www.affinity-sensors.com*), Intersens Instruments (Amersfoort, Netherlands), BioTul (*http://www.biotul.com*), and Artificial Sensing In-

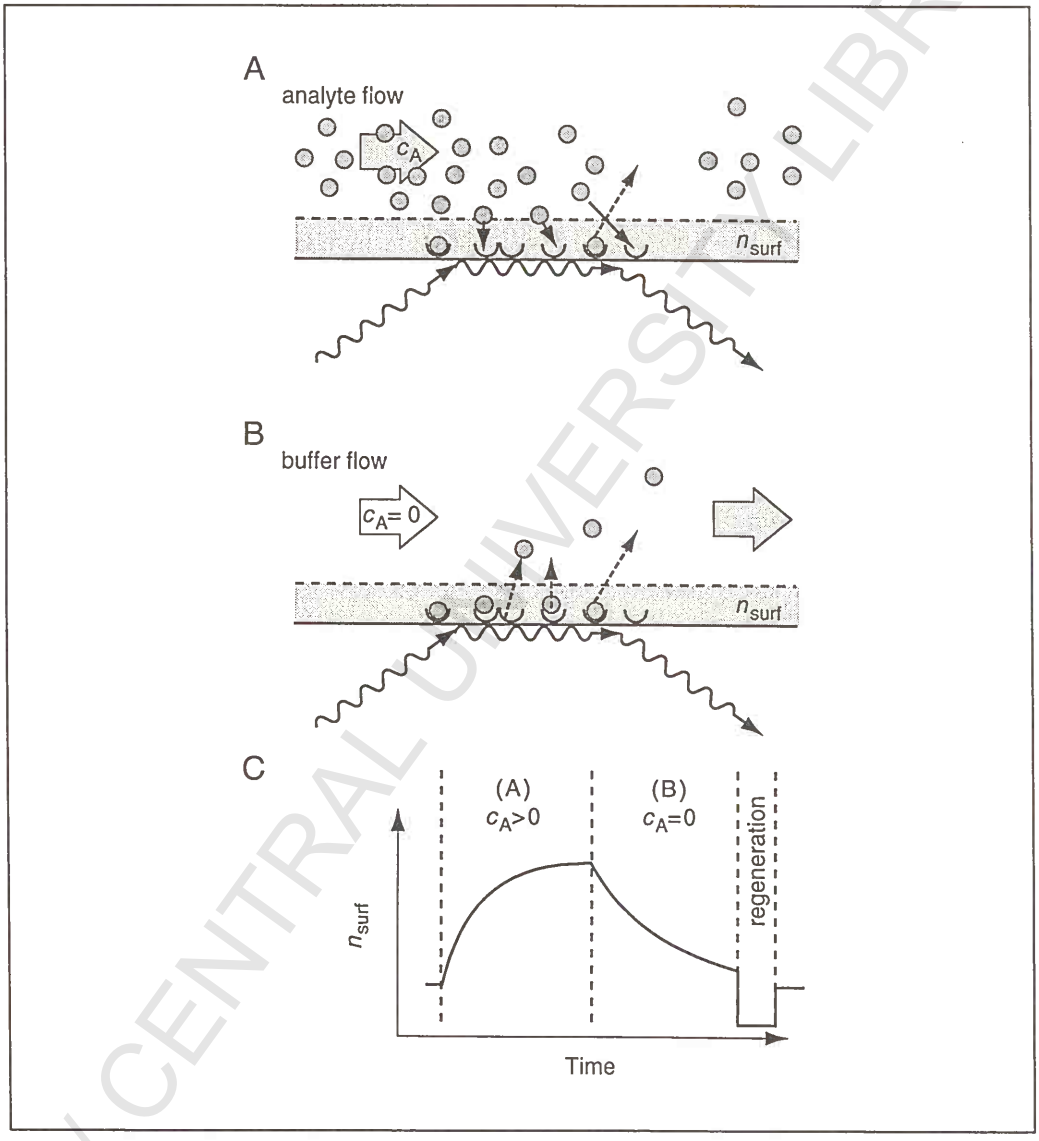

Figure 20.2.1 Schematic presentation of a typical optical biosensor experiment. Light is coupled into a structure that allows generation of surface-confined electromagnetic waves (e.g., surface plasmons in a gold film, or modes of a planar waveguide), which are sensitive to the refractive index of the solution in the range of the evanescent field, n_{surf}. Typical penetration depths of the sensitive volume into the solution are in the order of 100 nm. Ligands are attached to the sensor surface, as indicated by half-circles. (**A**) When analytes (full circles) are introduced into the solution above the surface, reversible interactions with the ligand lead to association events at a rate governed by $k_{on}c_Ac_{L,free}$ (solid arrows), and dissociation events at a rate $c_{complex}k_{off}$ (dashed arrows). In the association phase, association events outnumber dissociation events until a steady state is reached. (**B**) When the surface is washed with running buffer in the absence of analyte, only dissociation events take place. (**C**) Signal obtained from probing the refractive index n_{surf} during the sequential application of the configurations depicted in A and B, given in arbitrary units. Following the association phase (A) and the dissociation phase (B), usually a regeneration procedure is applied for removing all remaining analyte from the surface before a new experimental cycle takes place.

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struments (ASI AG, Zürich, Switzerland). The purpose of this unit is to provide a brief introduction to these techniques for the quantitative characterization of biomolecular interactions.

At the time of the introduction of optical biosensors in biomedical research, these instruments were thought to offer a particularly simple technique for the rapid quantitative measurement of equilibrium binding constants as well as binding kinetics. The reader is cautioned that the apparent simplicity and rapidity of the method is deceptive, and that the ease of using some of the commercial data analysis software packages can be highly misleading. The reliability of many of the initially used approaches in this field could not be verified in subsequent critical assessments of the methods. However, the technique has evolved significantly during the last few years. This is mainly due to the refinement and increasing variety of immobilization strategies, the identification of sources of artifacts, the use of control experiments, and the maturation of the analytical techniques (most notably global modeling). Emphasis is given in this unit to introducing the reader to robust approaches that will result in reliable results. Although the technique is frequently presented as being label-free, one of the interacting proteins has to be attached in an active form to the sensor surface. Most frequently, this is accomplished through covalent coupling, which can introduce all the problems well-known in chromophoric labeling and related covalent protein modifications. For all these reasons, the use of this technique requires careful planning and the execution of a number of different experiments, with time and effort more or less comparable to any of the other techniques available for the study of protein-protein interactions.

Optical biosensors are very versatile for the study of protein interactions. However, as described below (see Strategic Planning), the choice of the experimental strategy is usually dictated by the proteins under investigation. Only general guidelines can be given for this stage of the experiment. Detailed procedures for some of the most commonly employed immobilization procedures are described, followed by an introduction to the different strategies for the binding studies, and a brief description of some of the most commonly encountered problems. Next, the need for control experiments is emphasized, and the execution of a variety of such control experiments is described. Finally, some ideas are given for simple troubleshooting. Obviously, this introductory unit cannot be even remotely complete, or cover many of the more advanced techniques such as assembly of protein complexes (Andersen et al., 1999; Schuster et al., 1993). For many of these, the reader will be referred to the specialized literature cited throughout this unit.

STRATEGIC PLANNING

The first step in planning a biosensor experiment for the measurement of the interaction of two proteins requires the choice of the binding partner to be immobilized and the type of binding experiment that will be employed (steady-state, kinetic, or competition experiment, see Binding Experiments and Data Analysis). This involves the consideration of several factors related to size, purity, and chemical properties of the protein, as well as the stoichiometry of the interaction. In general, this will require the protein samples to be well characterized with respect to these properties.

Size, Concentration, and Volume

Optical biosensors detect changes in the refractive index at the sensor surface due to analyte binding. This signal is superimposed by an offset from the bulk refractive index differences of the buffers used). Because the refractive index increment of proteins is very similar in units of weight concentration, the signal obtained from saturation of a given

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number of surface sites will increase with increasing molar mass of the soluble protein (or other analyte). In some of the commercial instruments, the binding of an analyte with a molar mass of only 1000 Da can be detected without difficulty, given the commonly achievable surface densities of sites. There is virtually no upper limit for the size of the analyte. Size consideration is important before setting up the experiment if the two binding partners to be studied are of significantly different size. The choice of a larger species as the analyte is generally preferable because this provides a larger signal. However, the immobilization of a very small peptide is usually difficult to control, and in practice frequently leads to unsuitably high density of surface sites, which can introduce problems of steric hindrance and mass transport limitation (see discussion of Mass Transport Limitation). In the absence of any other considerations, it seems advantageous to immobilize the larger binding partner, in order to diminish the probability of steric hindrance problems brought about by binding to surface sites in close vicinity. This will also help to minimize the probability of the immobilization affecting the conformation of the binding epitope. On the other hand, peptide ligands often are far more resistant to the denaturing effects of repeated cycles of binding and regeneration, and can be expected to provide a more stable binding surface than most proteins.

If one of the proteins under study is available only in small quantities, it is advantageous to immobilize this species, because during the course of a study usually only a few functionalized surfaces are needed, each requiring usually only a few micrograms of material. The analytes are used in larger amounts; in one complete set of kinetic experiments volumes of the order of 100 µl at concentrations of ten times the K_D are typically required. In competition experiments, similar quantities of the immobilized species are additionally required. The active concentration of the analyte should be well known, because errors in this value will directly translate into proportional errors of both the binding constant K_D and the chemical on-rate constant k_{on}, resulting from analysis of the experiment (see Binding Experiments and Data Analysis).

Purity

The sample purity requirements in biosensor experiments can be relatively low. In principle, the criterion for sufficient purity is the absence of cross-reaction between the two samples, aside from the interaction of the proteins under study—i.e., that no binding occurs between any contaminating species in the ligand sample and in the analyte sample. In practice, this means that at least either one of the samples should be highly purified. However, very strict requirements have to be met to ensure the absence of contaminating multimeric aggregates of the mobile species (see discussion of Analyte Aggregates, below, and Davis et al., 1998).

Oligomeric Structure, Stoichiometry, and Multivalent Binding

It is important to avoid multiple attachment of a single analyte molecule to two or more surface sites. If this occurs, the lifetime of the surface-bound complex is greatly enhanced. This avidity effect depends strongly on the local density of surface sites (Muller et al., 1998), and the binding data obtained under these conditions are governed to a large extent by the specific properties of the functionalized surface, much more than the surface binding properties of the analyte. (It cannot be assumed that the avidity effect of multivalent binding to the sensor surface is similar to possible avidity effects in binding to cell surface receptors.) Therefore, if the proteins under study are subject to multimeric binding, the multivalent species must be chosen for immobilization. For example, in antibody-antigen interactions, the antibody should be immobilized and the antigen should be the mobile analyte (Fig. 20.2.2). For unknown stoichiometries, appropriate control experiments should be performed that can identify the presence of multivalent species.

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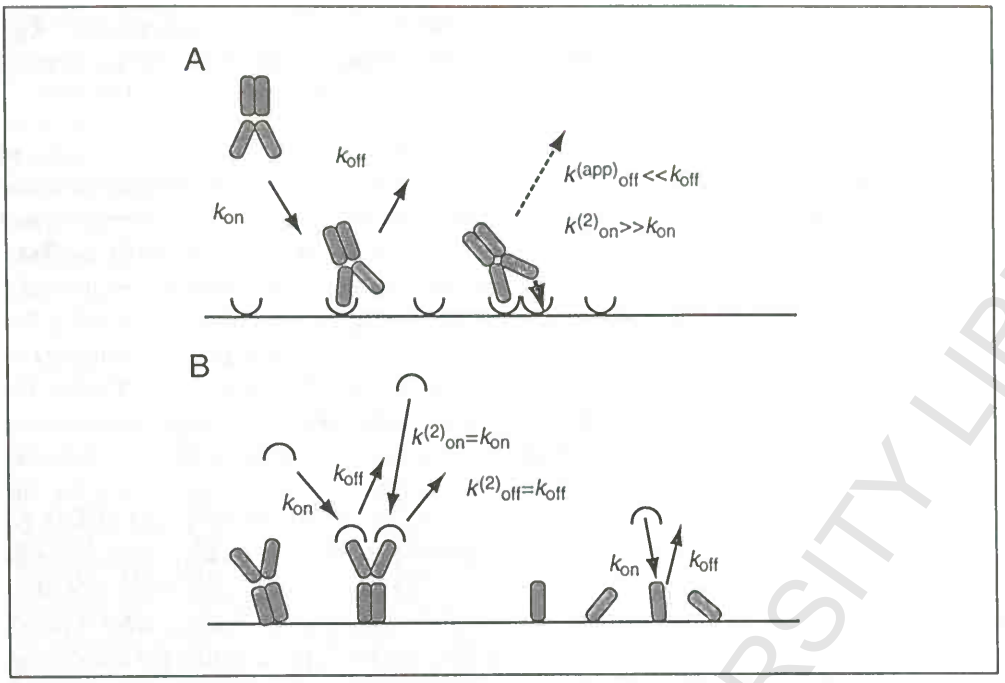

Figure 20.2.2 (**A**) The binding of a bivalent analyte can take place through the interaction with a single ligand molecule (left), or with two ligand molecules (right), depending on the local ligand density. If an unoccupied immobilized ligand molecule is within an accessible distance to an already singly bound analyte molecule, then the on-rate constant of the second interaction is strongly enhanced because of the entropic colocalization constraints. The overall dissociation rate constant of the double attached analyte is substantially reduceed (usually by orders of magnitude), because it requires the virtually simultaneous dissociation of both interactions, which is much more improbable than a single dissociation event. The overall binding kinetics obtained from multivalent analytes is highly complex. (**B**) If the configuration is reversed, with the bivalent binding partner immobilized to the sensor surface, then both valencies act independently (left), and (in the absence of cooperativity of the sites) lead to binding kinetics that are identical to that of the monovalent interactions (right).

For the same reason, the presence of even very small amounts of reversibly formed oligomers (self-association) or irreversibly formed oligomers can significantly interfere with the analysis of surface binding equilibria or kinetics. Because of the difficulties in detecting relatively weak or rapidly reversible oligomerization, solution techniques like analytical ultracentrifugation can be preferable to chromatographic or electrophoretic techniques.

Reactive Properties: Susceptibility to Immobilization, Nonspecific Binding, and Regeneration

The key to successful biosensor experiments is the immobilization of one protein species in an active, and, if possible, uniformly oriented state to the sensor surface. The ease of immobilization can be the major consideration for the choice of the immobilized species. Examples are the presence of biotin, histidine, or other tags on the molecule that allow specific capture, or the presence of a single accessible cysteine suitable for employing specific cross-linking chemistry. If a protein is produced by recombinant methods, appropriate tags can frequently be introduced.

Analogously, the protein that exhibits less nonspecific surface binding is the preferable choice for an analyte. Many sensor surfaces are slightly hydrophobic, and it is highly recommended to measure the extent of binding of both proteins to the nonfunctionalized surface at the maximal concentrations to be used in the binding experiments (e.g., at

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concentrations equal to 10-fold the expected dissociation equilibrium constant, K_D). Significant degrees of nonspecific binding can make the analysis of the surface binding kinetics very difficult.

Most analytical strategies require a series of multiple association/dissociation cycles to be executed with the same surface. Therefore, conditions need to be established that allow removal of all surface-bound analyte, without permanently altering the conformation and activity of the immobilized ligand (but tolerating possible destruction of the analyte). Naturally, the choice of the regeneration procedure depends very much on the proteins. Frequently, helpful hints can be obtained from the procedure used to purify the ligand—e.g., conditions employed during ion-exchange or affinity chromatography. Examples of common regeneration procedures include exposure to low-pH glycine or HCl buffer for 1 or 2 min. For some sensitive ligands that interact weakly with their respective analytes, an extended washout with the binding buffer is the gentlest and most reliable regeneration procedure. After selection of a regeneration procedure, control experiments for the integrity of the surface and for the reproducibility of the observed signal should be performed. These should consist of several identical association/dissociation/regeneration cycles. Insufficient regeneration can be identified by residual signal increase after each cycle and/or a negative drift of the baseline. The loss of binding sites through too-harsh regeneration conditions is indicated by a maximal signal during the association phase, which decreases with each cycle. Regeneration is not required for the equilibrium-titration techniques (Schuck et al., 1998; Myszka et al., 1998).

IMMOBILIZATION PROTOCOLS

The goal of immobilization is the stable coupling of the ligand to the sensor surface in an active form. In addition to the obvious functional test of analyte binding during the course of the interaction study, probing the conformation with monoclonal antibodies against ligand epitopes can be highly useful. If possible, it is desirable to reverse the role of ligand and analyte to rule out artifacts of immobilization on the interaction. It is important to note that the immobilization methods differ in their requirement for the exposure of the ligand to relatively harsh conditions, such as pH values below the pI in the commonly employed amine coupling. A related consideration in the choice of the immobilization strategy is the orientation of the surface-immobilized ligand. Random coupling techniques, such as amine coupling, may create different subpopulations of differently reactive ligand molecules at the surface, while specific orientation techniques (e.g., through specific cysteines or through specifically attached biotin moieties) create uniform surfaces. An elegant method for allowing uniform surface attachment is the introduction of specific tags (such as cysteine or histidine) into recombinantly produced or synthetic ligands.

Below are basic descriptions of three of the most commonly used immobilization techniques, which can be applied to the most commonly employed carboxymethylated dextran sensor surfaces. In any of these methods it is very important to control the number of surface sites, since several surface-related artifacts are invoked if the surface density of the proteins under study is too high. The density of immobilized protein can be measured as the difference of the signal before and after immobilization, which can be transformed into the maximal analyte binding capacity by multiplication with the molar mass ratio of the analyte and ligand. For kinetic experiments, generally a relatively low density of immobilized protein should be obtained—i.e., a binding capacity of 50- to 200-fold of the instrumental noise [~50 to 200 units (RU) on a Biacore system]. For competition and for certain control experiments (see Competition Analysis), a larger binding capacity can be desirable. In practice, for initial orientation on the behavior of a particular system of interacting proteins, it is best to prepare several different surfaces of

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different ligand densities and to compare the analysis of each. This will be helpful for diagnosing the regimes where mass transport limitations may be present (see Mass Transport Limitations), or where high surface coverage may lead to steric hindrance of analyte binding to neighboring surface sites (leading to biphasic surface binding kinetics).

Instruments that allow the on-line observation of a reference surface can be optimally utilized if the reference surface is treated as similarly as possible to the functionalized surface. This is important predominantly because of possible nonspecific interactions of the analyte with the sensor surface, and the possible changes in surface properties due to immobilization. For amine coupling, this can include the application of the activation/deactivation cycle to the reference surface, but without cross-linking of any protein, or with cross-linking of a nonfunctional control protein (for example an unrelated antibody of the same isotype). For the same reason, if using biotin-streptavidin coupling, it is advantageous to immobilize streptavidin to both surfaces.

A variety of additional immobilization techniques are available in addition those described below, such as hydrazide coupling (O'Shannessy et al., 1992), Ni^{2+} chelate coupling (Gershon and Khilko, 1995; O'Shannessy et al., 1995; Sigal et al., 1996), coupling of hydrophobic groups (Stein and Gerisch, 1996), the use of self-assembled monolayers (Sigal et al., 1996) or supported lipid or hybrid bilayers (Plant et al., 1995; Ramsden et al., 1996), and immobilization to aminosilane-derivatized surfaces (Edwards et al., 1995; Buckle et al., 1993). For details on chemical modifications of the proteins, see Hermanson (1996). Additionally, capturing strategies can be used for reversible attachment of the ligand. More specific information on commercially available sensor surfaces, and step-by-step protocols for the corresponding immobilization techniques, can be found in the commercial instrument handbooks, such as the Biacore applications handbook or the IAsys applications notes.

Amine Coupling

Amine coupling is most frequently carried out using a sensor surface that is modified with carboxymethylated dextran. It relies on the electrostatic preconcentration of the ligand to the sensor surface, and therefore requires that the proteins be positively charged. The coupling buffer should be 1 to 2 pH units below the pI of the protein, of low ionic strength (to avoid charge screening), and not contain any primary amines. Effective pre-concentration can be tested before the activation of the surface. It is frequently necessary to test immobilization with several different buffers of slightly different pH; an adjustment by only one half pH unit can be crucial for a successful immobilization.

In a typical protocol, the carboxyl groups of the sensor surface are activated by exposure for 5 to 7 min with 50 mM *N*-hydroxysuccinimide (NHS) and 200 mM *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide (EDC). Next, the ligand is injected in 10 mM sodium acetate pH 4.5 buffer. To control the amount of cross-linked ligand, it is usually better to adjust the reaction time as compared to adjustment of the ligand concentration. Repeated injection/wash cycles can be used to differentiate the covalently attached ligand from electrostatically surface-bound ligand. Finally, the surface is deactivated with 1 M ethanolamine·HCl, pH 8.5, for 7 min. Biacore and IAsys provide kits and prepared buffers for this procedure. The NHS and EDC should be kept frozen separately, in aliquots, at –20°C until use.

Avidin- or Streptavidin-Biotin Coupling

Covalent immobilization of avidin or streptavidin allows the capture of biotinylated proteins. This method avoids the need to expose the protein to a pH lower than the pI, and therefore can better preserve activity. Procedures for biotinylating the analyte are found

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in Hermanson (1996). The affinity of biotin-avidin interaction is very high and leads to a very stable surface. It should be noted that streptavidin-coated surfaces tend to be more sticky and lead to an increased level of nonspecific binding of the analyte to the surface. Therefore, good blank control surfaces are important.

The amine coupling protocol described above and a carboxymethylated dextran surface can be used for this procedure. The surface is activated with NHS/EDC (see discussion of Amine Coupling), the streptavidin (~100 μg/ml in buffer of pH 4.5 to 5.0) or avidin (~50 μg/ml in pH 4.5 buffer) is immobilized, and the surface is deactivated with ethanolamine. Note that, if commercially prepared streptavidin surfaces are used, this activation step can be omitted. The biotinylated protein is then injected; usually concentrations of 10 to 100 μg/ml and low flow rates are sufficient for efficient capture. Typical times for exposure of the surface are 10 to 30 min.

Thiol-Disulfide Exchange Coupling

This immobilization technique is frequently used for acidic proteins, for small peptides, and for cases when immobilization in a specific orientation is important. It also serves as a highly efficient coupling procedure that can be used if amine coupling or avidin-biotin coupling is unsuccessful. In this method, the immobilization is accomplished either by the introduction of an active disulfide group onto the sensor surface, which can react with thiols on the protein to be immobilized, or, vice versa, by the modification of the sensor surface with a thiol group, which then can react with active disulfides on the protein. The choice between these alternative procedures is dependent on the protein, i.e., the presence of accessible thiol groups, or the potential for introducing activated disulfide groups. The latter procedure may be accomplished, e.g., using 2-(2-pyridinyldithio)ethaneamine (PDEA) to derivatize carboxyl groups or succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT) for derivatizing amino groups. It should be noted that, in general, thiol-disulfide exchange coupling cannot be used for studies in the presence of reducing agents. However, the use of covalent thioester coupling—e.g., with succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), sulfo-SMCC, or *N*-(γ-maleimidobutyryloxy)succinimide ester (GMBS)—produces a surface that is stable against reducing agents, and allows one to specifically chose the site of coupling. In addition, the latter technique provides a small spacer between the protein and the surface, which may allow better accessibility (Khilko et al., 1995).

BINDING EXPERIMENTS AND DATA ANALYSIS

Optical biosensors provide data on the time course of analyte binding, and allow for a number of different experimental and analytical strategies for interaction analysis. Most rely on a repeated cycle of association, dissociation, and surface regeneration (Fig. 20.2.1). Among the most important analysis strategies are the kinetic analysis, which takes advantage of the real-time observation to obtain the kinetic rate constants of the reaction; the steady state analysis for measuring the affinity of the reaction; and the competition analysis for measuring the affinity of the interaction in solution. The choice may be constrained by the affinity and kinetics of the proteins under study. If possible, multiple approaches should be taken and the results should be compared for their consistency. Their optimal application requires approximate knowledge of the order of magnitude of the affinity of the interaction. This may make it necessary to perform these studies in several steps.

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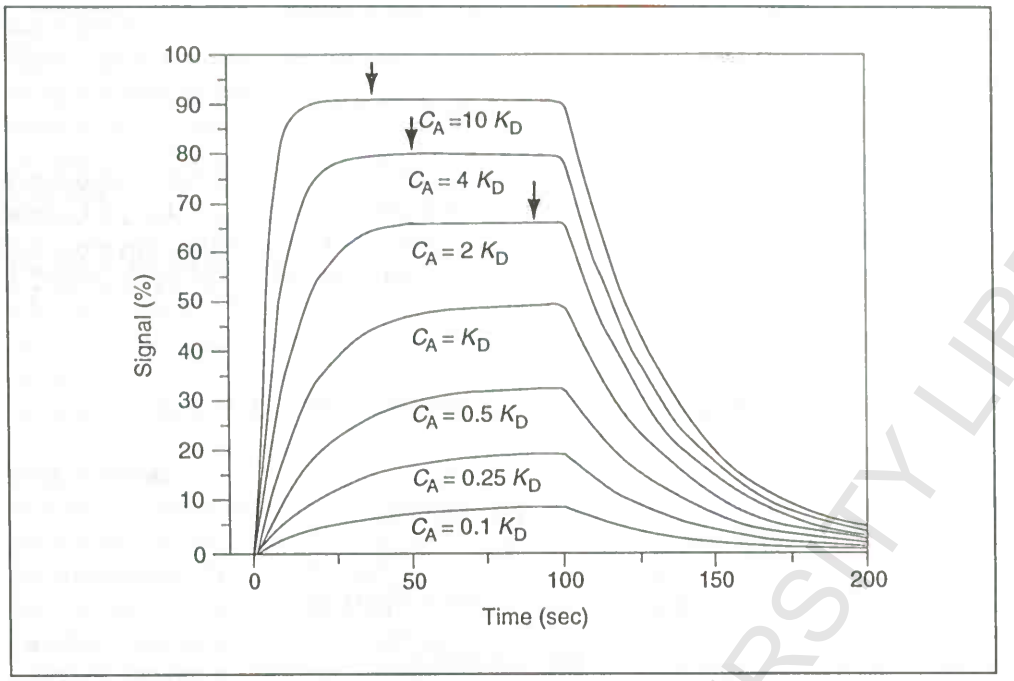

Figure 20.2.3 Schematic presentation of an ideal 1:1 pseudo first-order reaction. Shown is a superposition of the binding progress curves that should be obtained in a series of experiments at different analyte concentrations. The data are given in percent of the signal at maximal binding, but the units of the signal do not affect the results of the data analysis. The arrows on the curves at the higher concentrations indicate that steady-state binding is attained, whereas, for the lower curves, longer association times would be required as part of a steady-state analysis.

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Kinetic Analysis

Experimental

The experiment consists of repeated injections of the analyte at different concentrations (Fig. 20.2.3). It should be conducted at relatively low surface density of sites, to avoid kinetic artifacts arising from mass transport limitation. An analyte concentration range as wide as possible should be covered (at least from 1/10 of the K_D to 10 times the K_D), using two-fold or three-fold dilutions. The time allowed for the surface binding phase (injection time) should be long enough for the observation of significant curvature in the binding progress, since it is only the curvature that contains the information about the reaction rate constants. It is highly desirable that steady-state binding be attained, at least for the highest concentrations used. The observation time for the dissociation phase should at least be similar to the injection time.

Data analysis

If the analyte-ligand interaction follows a pseudo first-order reaction, L + A ⇔ LA then the measured binding progress (proportional to [LA]) is described by:

$$\frac{dR}{dt} = k_{on} c_A (R_{max} - R) - k_{off} R$$

Equation 20.2.1

where c_A denotes the analyte concentration, R denotes the signal (in arbitrary units), and R_{max} denotes the signal at maximum saturation of the surface sites. The goal is the calculation of the chemical on-rate and off-rate constants, k_{on} and k_{off}, which relate to the equilibrium constant as:

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$$K_D = \frac{k_{off}}{k_{on}}$$

Equation 20.2.2

While it is possible, in principle, to transform the data as dR/dt versus R in the association phase and as log(R) versus time in the dissociation phase, where $k_{obs} = (k_{on}c_A + k_{off})$ and k_{off}, respectively, would represent the slopes of the obtained straight lines, this procedure is very unreliable. Instead, it is state-of-the-art procedure to directly fit Equation 20.2.1, or its integrated form:

$$R_{assoc}(t) = \frac{R_{max}}{1 + \frac{k_{off}}{k_{on}c_A}}[1 - \exp(k_{on}c_A + k_{off})t]$$

Equation 20.2.3

$$R_{dissoc}(t) = R_{assoc}(t_d)\exp[-k_{off}(t - t_d)]$$

Equation 20.2.4

directly to the data (with t_d denoting the end of the association and the beginning of the dissociation phase, where $c_A = 0$; O'Shannessy et al., 1993). This should be done without truncation of the data beyond possible regions of artifacts from buffer changes or injections. Global analysis should be employed, which allows one to analyze all experimentally observed curves at all analyte concentrations simultaneously. This results in unambiguous best-parameter estimates for the binding constants, and provides residuals that should be inspected for comparison of the model and the data. Software needed for global analysis can be obtained from the manufacturer or as shareware. It should be noted that exclusion of specific data regions that may be poorly described by the model will introduce a bias into the analysis.

If the global analysis cannot be performed, and separate analyses of the individual binding curves of each experiment according to Equation 20.2.3 and Equation 20.2.4 are applied, then tests for consistency of the results are crucial (Schuck and Minton, 1996a). Important tests are: (1) the estimated extrapolated values of R_{assoc} ($t \rightarrow \infty$) at different concentrations should be consistent with a Langmuir isotherm for the estimated K_D (e.g., approaching half-saturation at $c_A = K_D$; see Figure 20.2.2); and (2) the extrapolated values of ($k_{assoc}c_A + k_{off}$) from the association phases should be consistent with the value of k_{off} as estimated in the dissociation phase. An analysis of the accuracy of the kinetic rate constants can be found in (Ober and Ward, 1999a).

Possible problems

The data may be only poorly described by Equation 20.2.3 and Equation 20.2.4 (leading to a poor fit and systematically distributed residuals) and instead show multiphasic behavior. Although it is in principle possible to apply global modeling with multiple binding-sites models, this is not recommended, because it is very difficult and can easily lead to very large errors (up to several orders of magnitude). Such modeling is usually not very robust, and many different models may fit the same data equally well (Glaser and Hausdorf, 1996). The reader is also advised to be extremely cautious with some of the implemented models in commercial or shared software, because they may not be valid descriptions of real surface binding processes (for example, mass transport models or models for bivalent analytes). Frequently, the deviations of the data from the single

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exponential binding progress predicted in Equations 20.2.3 and Equation 20.2.4 are signatures of experimental artifacts, such as heterogeneity of the ligand (subpopulations of ligand with different binding properties through nonspecific immobilization), steric hindrance of binding to neighboring sites, or the presence of mass transport limitations or multivalent aggregates of the analyte (see discussion of Common Experimental Obstacles).

Steady-State Experiments and Binding Isotherm Analysis

This approach can yield both equilibrium constants and, if appended by a dissociation analysis, kinetic constants. Its main advantage with respect to the previously described technique (see discussion of Kinetic Analysis) is that it is much more robust and reliable because it is less affected by problems of potential multiphasic binding.

Experimental

If in the course of the association experiments a steady-state signal is observed, which is generally possible for interactions with relatively high k_{off}, then a binding isotherm can be constructed (Figs. 20.2.3 and 20.2.4). In an experimental procedure similar to the kinetic analysis, a concentration range as wide as possible should be covered, with two-fold concentration steps starting from both far below ($\leq 1/10$) K_D up to almost complete saturation of the surface sites, far above the K_D (≥ 10 times the K_D). The injection times required to attain steady state at the lower concentrations are longer (see Equation 20.2.3 and Equation 20.2.4), and exponential extrapolation may be used, in particular in the Biacore system, since the association times may be limited in the by the limited volume in the injection loop of the microfluidics. Exponential extrapolation is not advised, however, for concentrations above K_D.

For interactions that do not yield steady-state binding within a tolerable time interval for the association phase, or where the ligand does not withstand a regeneration procedure, equilibrium titration represents an experimental variant (Fig. 20.2.4). In this approach, the analyte concentration is increased in two-fold steps, allowing for attainment of steady state in each step, avoiding regeneration between the steps. This can be achieved by addition of a small aliquot of the analyte into a fixed volume of solution above the sensor surface (in cuvette-based sensors; Hall and Winzor, 1997), into a loop of recycling sample (slightly modified Biacore X; Schuck et al., 1998), or into the running buffer reservoir of a standard Biacore (Myszka et al., 1998). The fixed-volume techniques require far less sample volume.

After the highest analyte concentration has been applied, the dissociation phase can be observed and analyzed in terms of Equation 20.2.4. This can give an estimate of k_{off}, which can be used in conjunction with Equation 20.2.2 to calculate k_{on} as k_{off}/K_D.

Data analysis

Although the data analysis could in principle be performed by Scatchard analysis, this is not recommended for statistical reasons. A better method is a plot of $R_{steady\text{-}state}$ versus $\log(c_A)$, in which the K_D can be easily estimated as the inflection point at half saturation of the typical sigmoid isotherm (Fig. 20.2.4). The functional form for modeling is:

$$R_{steady-state}(c_A) = \frac{R_{max}}{1 + K_D/c_A}$$

Equation 20.2.5

Even if the data cannot be fitted well with this expression, the 50% saturation will still represent a good estimate of the order of magnitude of an average K_D. It is important,

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however, in such an approach, to have enough data points at high analyte concentrations for a reliable estimate of R_{max} and the 50% saturation level, respectively. Then the robustness will be greater than that of the kinetic analysis of Equations 20.2.3 and Equation 20.2.4.

Competition Analysis

Competition experiments can be performed in different variations, but conceptually all use a calibration of the sensor signal as a function of free analyte concentration, followed

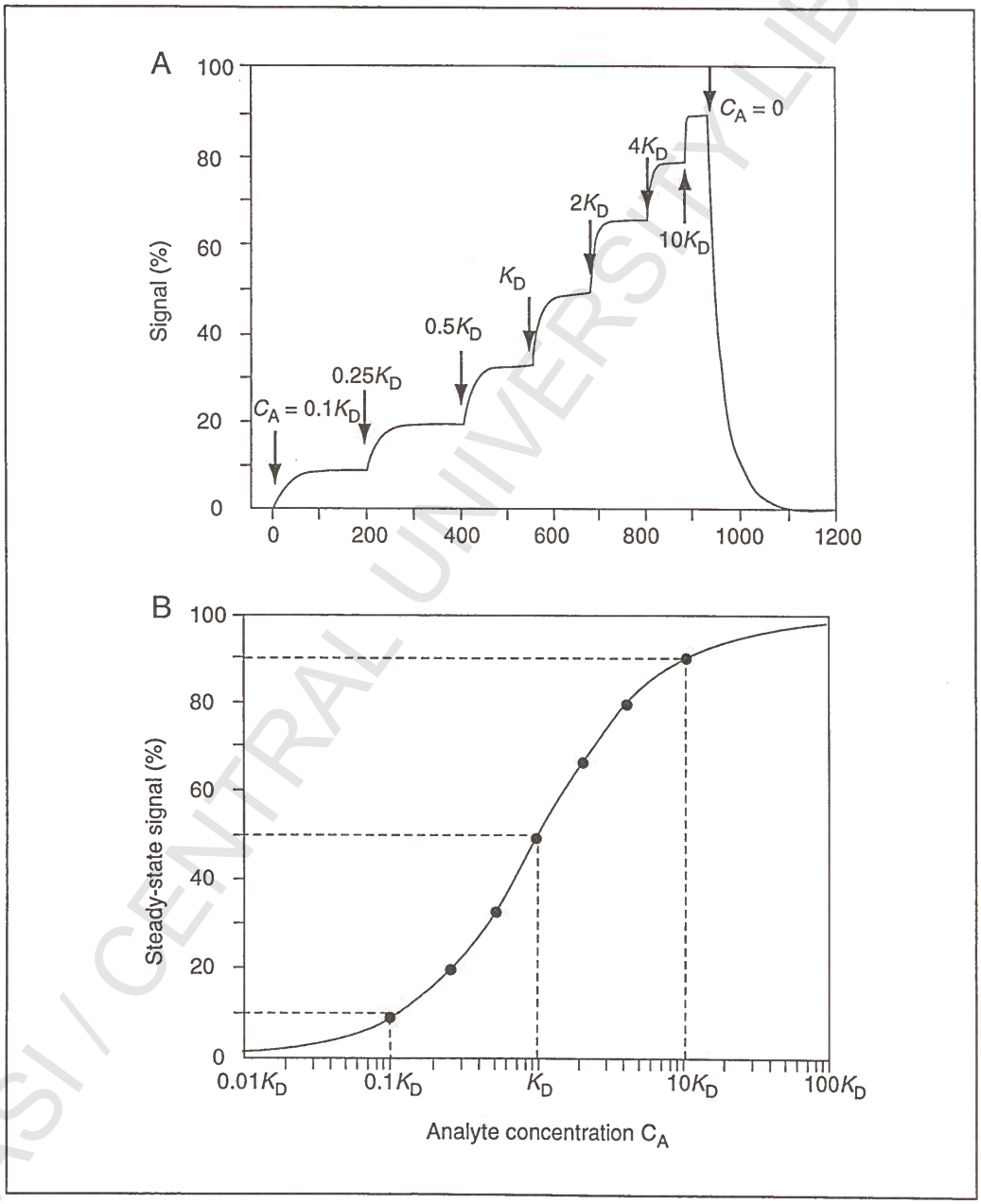

Figure 20.2.4 (**A**) Time course of an equilibrium titration experiment. Arrows indicate the time-points of the step-wise increase in the analyte concentration. (**B**) Steady-state binding analysis according to Equation 20.2.5. In a plot of the steady-state signal versus the base-10 logarithm of the analyte concentration, the binding isotherm exhibits a typical sigmoid shape. The inflection point at 50% saturation determines K_D, and the width of the curve is characterized by ~10% saturation at 1/10 K_D and ~90% saturation at 10 K_D (dotted lines). Visible inspection of the data in this representation already allows a robust parameter estimation.

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by experiments with mixtures of analyte and soluble ligand in different molar ratios. The basic presumption is that the soluble ligand competes with the immobilized ligand for the free analyte, which leads to a reduction of the surface binding. This gives information on the solution affinity of ligand and analyte, while the freedom from the need to explicitly model the surface binding drastically diminishes the influence of potential surface artifacts on the results (Nieba et al., 1996; Schuck et al., 1998). This analysis can be extended to any other competitor that completely inhibits ligand binding of the analyte when bound to the analyte.

Experimental

For low-affinity systems with high chemical off-rate constants, in general, steady-state conditions can be achieved. Accordingly, the binding isotherm can be taken as a "calibration" of the sensor signal as a function of the free analyte concentration. Experiments to derive the surface binding constant can be conducted as described above (see Steady-State Experiments and Binding Isotherm Analysis). In a second variation, for high-affinity systems, mass-transport limited conditions are established by using a large immobilization density. Under these conditions, the initial binding rate constant will be directly proportional to the free analyte concentration ($dR/dt = k_t c_A$). The initial binding rate constant should be measured in equidistant concentration intervals for analyte concentrations up to a few times (two-fold) the K_D. After either one of these sets of calibration experiments, a set of experiments is performed using a constant concentration of analyte, $c_{A,tot}$ (which should be taken in the range of K_D), premixed and equilibrated with different concentrations of the soluble form of the ligand. The ligand concentrations, c_L, should be taken in two-fold steps, spanning the range from far below to far above the K_D. Choice of the fixed analyte concentration is important, since too high a value for $c_{A,tot}$ would show competition only in the range of stoichiometric binding. For systems that do not withstand regeneration, a recycling competitive titration procedure has been developed (Schuck et al., 1998).

Data analysis

For the high-affinity variant of the experiment with a linear initial rate of binding, $dR/dt = k_t c_A$, a plot of dR/dt versus c_A can be used to graphically calculate the amount of free analyte, $c_{A,free}$, during the competition experiment, or, $c_{A,free}$ is calculated according to the analytical inversion $c_{A,free}(c_L) = dR/dt(c_L)/k_t$. Similarly, in place of the linear function, an empirical calibration function for $dR/dt(c_A)$ could be used. The obtained concentration of free analyte $c_{A,free}$ in experiments at different soluble ligand concentrations c_L can be plotted as $c_{A,free}$ versus $\log(c_L)$, which gives a competition isotherm with the characteristic sigmoid shape. It can be fitted with Equation 20.2.6, the solution of the quadratic equation of the mass action law combined with mass balance:

$$c_{A,free} = c_{A,tot} - \frac{1}{2}\left(c_{A,tot} + c_L + K_D^{sol} - \sqrt{(c_{A,tot} + c_L + K_D^{sol})^2 - 4c_{A,tot}c_L}\right)$$

Equation 20.2.6

which gives the solution binding constant of analyte and ligand, K_D^{sol}. If the low-affinity variant of the experiment was used, the steady-state analysis of the sensor response in the absence of soluble ligand can be performed according to Equation 20.2.5, giving R_{max} and $K_D^{surface}$. This can be used in a direct analysis of the competition steady-state response using:

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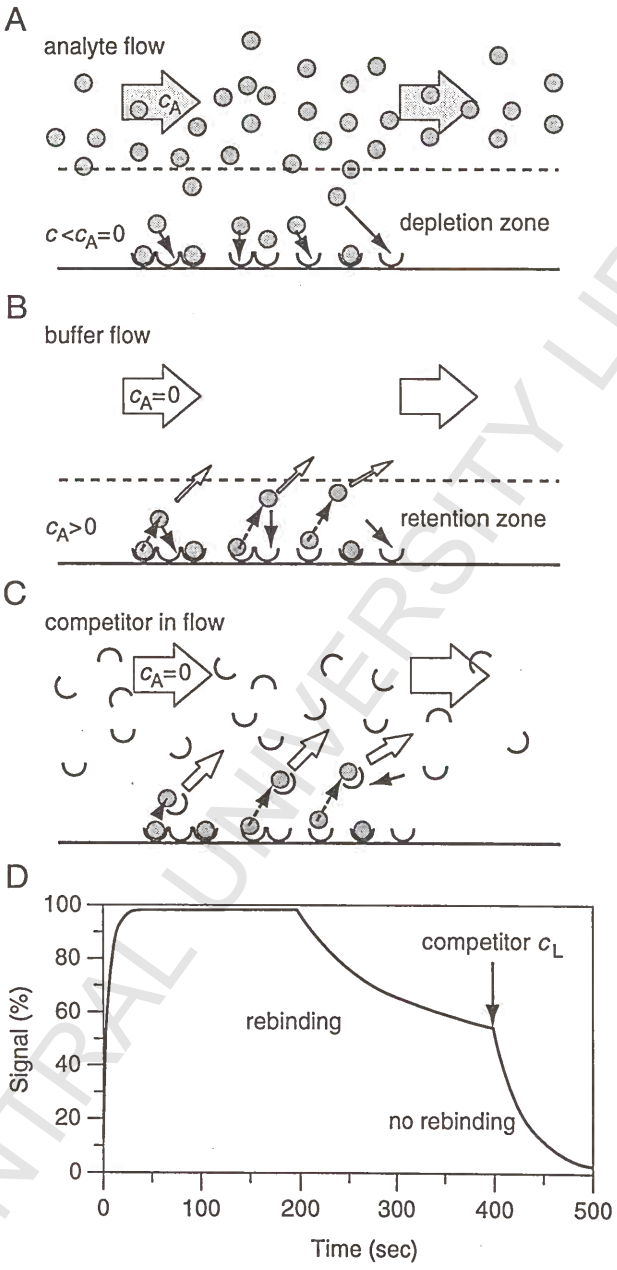

Figure 20.2.5 Mass transport effects on the surface binding process. (**A**) In the association phase, insufficient transport does not fully replenish the free analyte in a zone near the sensor surface (depletion zone). The inability to maintain the concentration c_A in the depletion zone leads to a limited surface binding rate. (**B**) The corresponding effect of mass transport limitation in the dissociation phase is that the surface is insufficiently washed and dissociated analyte is insufficiently removed. This generates a zone near the surface in which free analyte (after dissociation from the ligand) can rebind to empty ligand sites before diffusing into the bulk flow. This zone is indicated as retention zone. (**C**) Introduction of an excess of the soluble form of the ligand as a competitor into the buffer of the dissociation phase helps prevent rebinding to the surface. Soluble ligand can bind to dissociated analyte before rebinding takes place, and the soluble complex can diffuse into the buffer flow. This can allow the measurement of k_{off} free of mass transport effects. (**D**) Time course of dissociation during a mass transport induced rebinding situation (as depicted in panel B), and after introduction of a soluble competitor into the washing buffer (arrow). In the presence of the competitor, the signal reflects the chemical off-rate constant k_{off}, while in the rebinding situation, the apparent rate governing the signal is reduced.

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$$R(c_L) = \frac{R_{max}}{1 + K_D^{surf} / \left[c_{A,tot} - \frac{1}{2}\left(c_{A,tot} + c_L + K_D^{sol} - \sqrt{(c_{A,tot} + c_L + K_D^{sol})^2 - 4c_{A,tot}c_L} \right) \right]}$$

Equation 20.2.7

Alternatively, both titrations can be analyzed simultaneously in a global fit to both isotherms, assuming the binding constants to the surface sites and in solution to be identical.

COMMON EXPERIMENTAL OBSTACLES

Two of the most important experimental problems are mass transport limitations and the effect of aggregates on the binding kinetics. The first difficulty is found most frequently (but not exclusively) in systems of medium to high affinity with high k_{on}, whereas the second is observed predominantly in systems with lower affinity, where higher analyte concentrations are required. Other obstacles can be steric hindrance effects of binding to adjacent surface sites (O'Shannessy and Winzor, 1996), or the contribution of second-order kinetics to the binding process in cuvette systems (Edwards et al., 1998). Most of these processes scale with the immobilization level of the ligand. For this reason, a low ligand density is generally advantageous in minimizing potential problems. Special considerations for working at very low ligand densities are described in Ober and Ward (1999a,b). The use of control experiments (e.g., comparison of the K_D from solution competition experiments with the K_D obtained from surface binding kinetic measurements) is very useful in verifying the absence of artifacts. The role of mass transport and analyte aggregates will be explained in detail below.

Mass Transport Limitation

If the supply of analyte to the sensor surface is the limiting factor in the surface binding rate, the observed binding kinetics are mass transport limited. This will depend directly on both the density of the immobilized sites, and on the chemical on-rate constant of the interaction. In the association phase, this will produce a zone of depleted analyte concentration in the vicinity of the surface, while in the dissociation phase, this will lead to a zone of nonvanishing free analyte concentration, which is subject to rebinding-retention effect (Silhavy et al., 1975; Fig. 20.2.5). The quantitative influence of this process on the observed surface binding kinetics is governed by a highly complex reaction/diffusion/convection process (Yarmush et al., 1996; Schuck, 1996). Although the processes in the association and dissociation phase are conceptually closely related, it cannot be assumed that the ratio of the "apparent" rate constants still reflects a good estimate of the equilibrium constant. Also, it is strongly recommended that the nonspecialist reader refrain from modeling using mass-transport-corrected kinetic models.

Instead, the primary goal is to identify mass transport limitations, and to establish experimental conditions avoiding transport effects. The following is a list of diagnostic indicators, in the order of reliability, for mass transport limited binding.

1. *Dependence on surface capacity.* This is a very good indicator of mass transport limitation, since the transport limit always directly scales with the density of active surface sites. For this reason, variations of the immobilization density of the ligand are recommended as routine tests demonstrating the absence of transport limitation and other surface-related artifacts. This test can fail in the presence of significant non-specific analyte binding to the surface.

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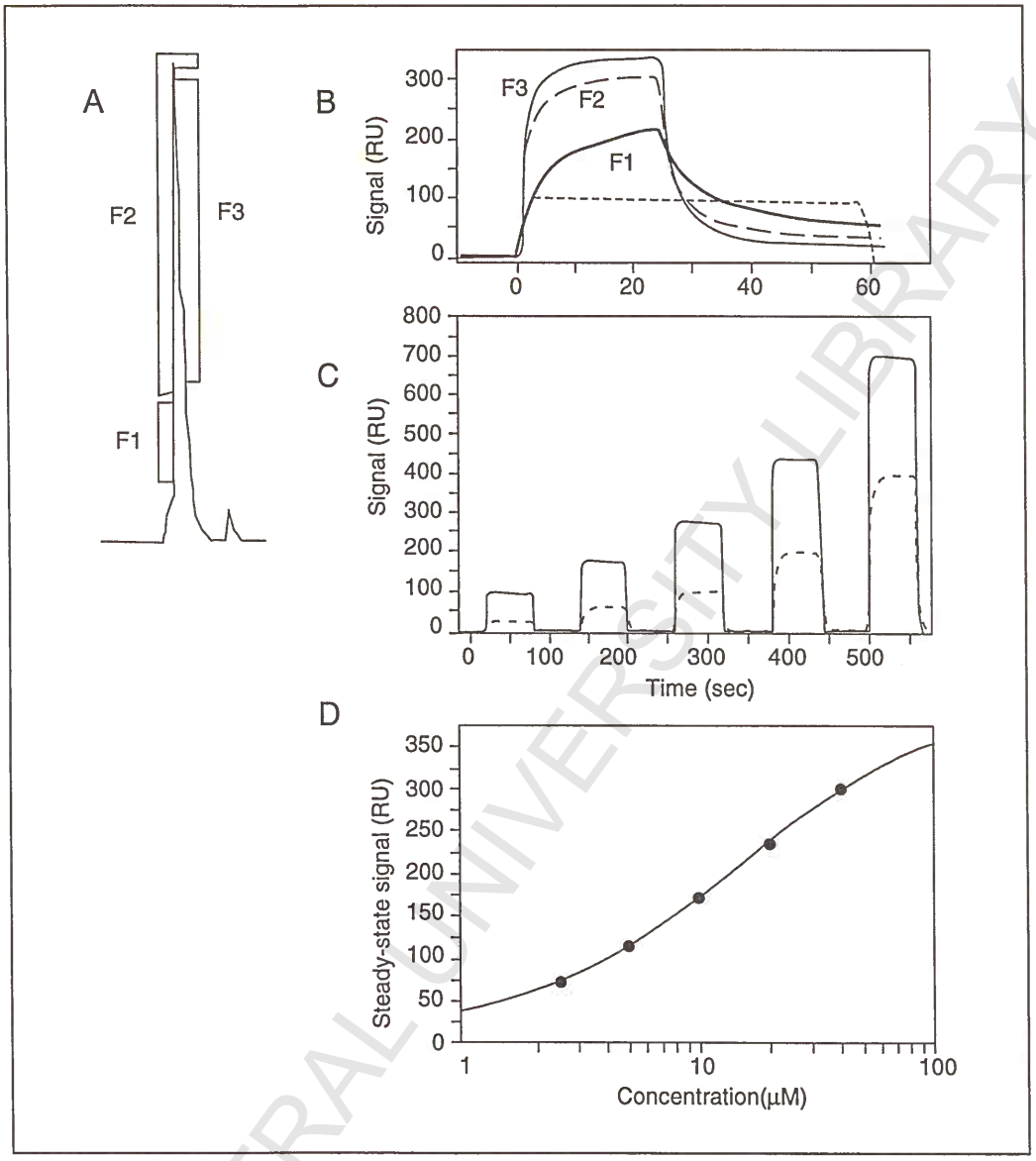

Figure 20.2.6 Exemplary data of the interaction of TCR with superantigen (for details of this interaction see Andersen et al., 1999). (**A**) Elution profile of the TCR from size-exclusion chromatography, indicating the fractions used for kinetic analysis of the interaction with immobilized superantigen, as shown in (**B**). It should be noted that fraction 1 (bold solid line), fraction 2 (dashed line), and fraction 3 (solid line) all exhibit multiphasic binding, with different relative magnitudes of the slower component. It should also be noted that despite the lower concentration of fraction 1, which results in the lowest response in the association phase, the signal in the dissociation phase is highest. This slower kinetic component in the binding progress curve reflects different amounts of aggregates bound to the sensor surface. The aggregates have a slower dissociation because of their multivalent attachment. For comparison, the same interaction is shown in the reverse orientation, with immobilized TCR and soluble superantigen (dotted line). (**C**) Sequence of association-dissociation curves at different superantigen concentrations, binding to immobilized TCR. In this orientation, the association and the dissociation is much faster, virtually monophasic, and the binding is completely reversible, which provides further evidence that the slow kinetic components introduced in the different fractions of the soluble TCR sample in Panel B are artifacts. For quantitative analysis, the signal measured at a nonfunctionalized surface (dotted line) is subtracted in order to remove the bulk refractive index contribution of the analyte. (**D**) A plot of the net steady-state binding signal versus superantigen concentration allows the measurement of the affinity of the interaction free from artifacts due to TCR aggregation that would be introduced in the configuration of Panel B.

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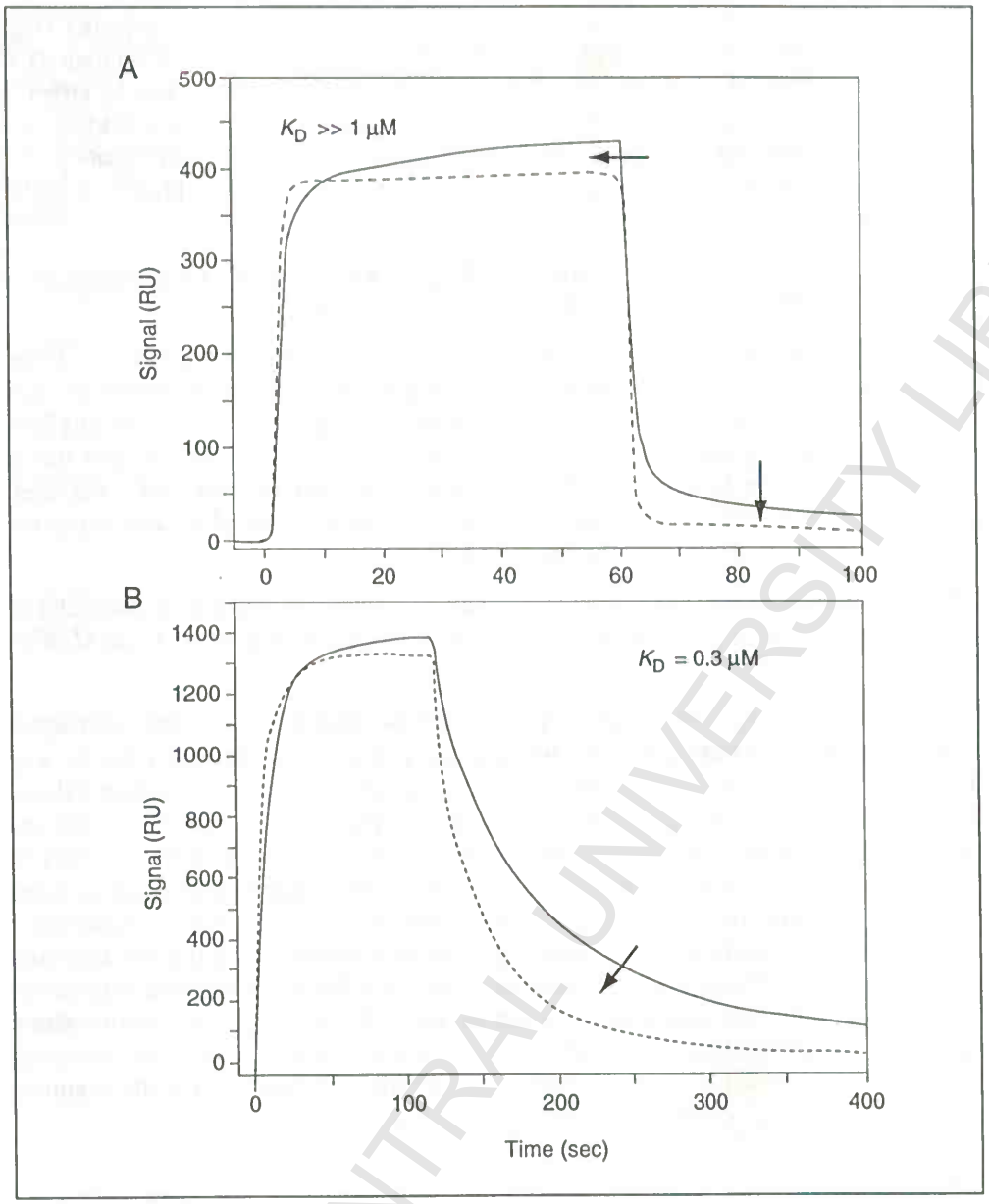

Figure 20.2.7 Effects of the ligand density on the binding kinetics for interactions with relative low affinity (**A**) and medium affinity (**B**). Each panel shows the interaction of immobilized superantigen with single-chain TCR, observed at high ligand surface density (1700 RU, solid lines) and low ligand surface density (700 RU, dashed lines). For easier comparison, the signal obtained at the lower density surface was scaled proportionally. In panel A, a slow phase of binding in the association phase and a residual binding (possible slow dissociation of multivalently bound aggregates) is introduced at high ligand density, under otherwise identical conditions. For the interaction in panel B, the chemical off-rate constant is smaller than for the low-affinity interaction shown above. Nevertheless, from comparison of the binding curves at different ligand surface densities (under otherwise identical conditions) it is obvious that an increased ligand density has significant effects on the surface binding kinetics. This observation could be due to aggregation or to mass transport limitations, both of which are more likely at higher ligand densities.

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2. *Increased dissociation rate when a soluble form of the ligand is injected (Fig. 20.2.5D).* This always strongly indicates rebinding (mass transport limitation) if detected. Since the ligand by itself should not interact with the surface, its effect is the binding to analyte near the sensor surface, preventing it from rebinding and allowing diffusion and washout from the surface (Fig. 20.2.5C). Unfortunately, this effect may not be present for large ligands or ligands/analytes with high nonspecific binding if they exhibit limited diffusivity.

3. *Double exponential dissociation phase.* This will be seen only after dissociation from more than 50% occupation of all available surface sites.

4. *Weak dependence on the flow rate (stirring speed in cuvette-based systems).* Since the transport parameters only change with the cube root of the flow rate, i.e., generating only a factor of 2 when changing the flow rate by a factor of 10, this flow rate dependency can be difficult to detect. This is particularly true if the flow rate is only varied by a factor of 2, and the reaction is only partially transport influenced. Then, only a ~10% change in the apparent k_{off} would be expected in cases where the true k_{off} is 100% larger than the apparent k_{off}.

5. *Linear association phase.* This effect may not always be present, in particular at substantial transport limitation (Schuck, 1996; Schuck, 1997b; Schuck and Minton, 1996b).

Generally, tests 1 and 2 are the most reliable. They also lead to experimental techniques that can be utilized to reduce or eliminate mass transport artifacts. The most effective way for reducing mass transport influence is lowering the surface density of the immobilized ligand. Higher flow rates give only comparatively very small improvements, but are connected with strongly increased sample volume requirements. If the surface density of the ligand cannot be reduced further without leading to an insufficient signal-to-noise ratio, then switching from kinetic experiments to steady-state or competition steady-state experiments is the best solution. This will give information on the equilibrium constant. The kinetic rate constants can then be estimated best from a saturation experiment (approaching complete saturation of all surface sites), followed by a dissociation phase during which soluble ligand is coinjected. The soluble ligand will minimize rebinding and allow the estimation of the chemical off-rate constant, from which the chemical on-rate constant can be determined via Equation 20.2.3.

Analyte Aggregates

Oligomeric aggregates of analyte can be troublesome in biosensor experiments in two different ways. First, if trace amounts of higher oligomers are present in the analyte sample, this will lead, in the association phase, to a slow accumulation at the sensor surface, which can be visible as a slower second phase of binding. As depicted in Figure 20.2.2, these multimeric analyte aggregates can have multiple interactions with immobilized ligand molecules, and therefore they will dissociate much more slowly than the monomeric analyte. Consequently, they will appear in the dissociation phase as a submoiety with a very low off-rate constant (Davis et al., 1998). The troublesome trace amounts of oligomers can be eliminated by careful chromatographic purification, or their influence can be minimized by exchanging the role of analyte and ligand (Davis et al., 1998; Andersen et al., 1999). This is illustrated in the example of Figure 20.2.6, which demonstrates the importance of both the sample preparation and the choice of ligand and analyte.

The second potentially problematic form of aggregation is a surface-induced multimerization of the analyte. Because the local macromolecular concentrations at the sensor surface are very high (e.g., in the order of 10mg/ml at a signal of 1000 RU in Biacore

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instruments), local crowding effects combined with non-specific interactions of the analyte can promote oligomer formation at the sensor surface (Fig. 20.2.7A; Minton, 1995, 1998). As with the influence of preformed aggregates, this process will lead to biphasic association and dissociation profiles, with the slower phase resulting from oligomer accumulation and dissociation, respectively. This process will be favored by higher surface concentrations—i.e., higher density of immobilized ligand, and by higher ligand affinity (Fig. 20.2.7). As with mass transport limitations, they can be detected by

Table 20.2.1 Troubleshooting Guide for Measuring Protein Interactions by Optical Biosensors

Problem	Solution
No electrostatic preconcentration achieved; poor immobilization	Desalt protein (e.g., using spin column or microdialysis); decrease pH of buffer used for immobilization (should be below pI of protein)
Nonspecific binding is high	Analyte may be too hydrophobic, or there may be electrostatic interaction with surface. Increase salt or detergent concentration in running buffer.[a]
High signal from buffer; refractive index changes	If this does not result from high analyte concentrations, dialyze the analyte against running buffer, or use a spin column for buffer exchange
Analyte does not come off after regeneration	Use increasingly harsher conditions for regeneration; test procedures used in affinity chromatography; check for strong nonspecific binding of the analyte; check for possible incomplete blocking of activated surface sites after immobilization
No binding of biotinylated sample to streptavidin surface	Check for presence of free biotin in sample. Biotin on the analyte may not be accessible by surface-immobilized streptavidin; in this case try biotinylated linker
Kinetics does not follow 1:1 binding	Check for mass-transport artifacts; check for possible traces of aggregates and for formation of aggregates at the surface; change immobilization method (avoid random coupling, avoid large surface densities). If this is not successful, go to steady-state analysis methods.
No steady-state binding is reached in a flow system	Use longer injections by increasing injection volume and/or decreasing flow rate. If sample volume is limiting, try an equilibrium titration
Mass transport limitation	Decrease immobilization density.[b] If immobilization density cannot be lowered further, go to steady-state analysis, combined with establishment of lower limit of k_{dis} from dissociation after saturation.
Increasing slope in the association phase	Potential signature of mass transport; lower the immobilization density
Increasing signal in the dissociation phase	Signature of mass transport limitation; lower the immobilization density

[a]Be aware of effects of nonspecific binding on the binding kinetics, which substantially decreases the diffusivity of the analyte across the sensor surface, potentially leading to mass transport artifacts that cannot be detected through change of the ligand density.

[b]Increasing the flow rate affects transport much less, but consumes much more sample.

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variation of the surface density of the ligand. They also can be reduced by lowering the surface density of the immobilized ligand, by size-exclusion chromatography immediately prior to the experiment, or by exchanging the role of ligand and analyte.

Troubleshooting

As with other complex biophysical techniques and all investigations involving biological samples, it is impossible to give guidelines which are even nearly complete or generally helpful for troubleshooting. Nevertheless, Table 20.2.1 presents a small list of possible solutions to potential problems, given in the hope that some readers may find them helpful.

SUMMARY

Binding studies with optical biosensors can be very powerful and versatile. Among the most important virtues are their high sensitivity and utility for a broad range of affinities, real-time detection allowing studies of binding kinetics, and relatively low requirements of sample volume. We have outlined some general strategies and described some of the most commonly used techniques. The work with protein interactions at a surface can introduce additional experimental difficulties as compared to solution methods. As a general rule in experiments with optical biosensors, it is highly recommended that analyses be performed in different ways so that the consistency of the results can be tested. Biosensors can be an excellent tool in the study of protein interactions, and become particularly powerful if combined with other methods.

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Davis et al, 1998. See above.

Contains a detailed description of analyte aggregation effects on the measured surface binding.

Nieba et al., 1996. See above.

Demonstration how competition approaches can be used to circumvent kinetic artifacts.

O'Shannessy et al., 1992. See above.

Collection of immobilization techniques.

Schuck, 1997b. See above.

General review of the method and its application.

Internet Resources

http://www.biacore.com

Web site for Biacore; extensive list of published biosensor applications

http://www.affinity-sensors.com

Web site for Affinity Sensorsl; extensive list of published biosensor applications.

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Common Conversion Factors

Table A.1D.1 lists some of the more common conversion factors for units of measure used throughout Current Protocols manuals, while Table A.1D.2 gives prefixes indicating powers of ten for SI units.

Table A.1D.3 gives conversions between temperatures on the Celsius (Centigrade) and Fahrenheit scales. Celsius temperatures are converted to Fahrenheit temperatures by multiplying the Celsius figure by 9, dividing by 5, and adding 32, or by multiplying the Celsius figure by 1.8 and adding 32. Fahrenheit is converted to Celsius by subtracting 32 from the Fahrenheit figure, multiplying by 5, and dividing by 9. In Table A.1D.3, the center figure represents the temperature one has read on one of the scales; the figure to the left is the conversion of that figure into Celsius if read in Fahrenheit, while that to the right represents the conversion to Fahrenheit if read in Celsius: e.g., the temperature 88 Fahrenheit converts to 31.1°C, while the temperature 88 Celsius converts to 190.4°F.

Table A.1D.1 Unit of Measurement Conversion Chart

To convert:	Into:	Use the multiplier:
amperes per square centimeter (amp/cm^2)	amperes per square inch (amp/in.^{2})	6.452
	amperes per square meter (amp/m^2)	10^4
amperes per square inch (amp/in.^{2})	amperes per square centimeter (amp/cm^2)	0.1550
	amperes per square meter (amp/m^2)	1.55×10^3
ampere-hours (amp-hr)	coulombs (C)	3.6×10^3
	faradays	3.731×10^{-2}
atmospheres (atm)	bar	1.01325
	millimeters of mercury (mmHg) or torr	760
	tons per square foot (tons/ft^2)	1.058
bar	atmospheres (atm)	0.9869
	dynes per square centimeter (dyn/cm^2)	10^6
	kilograms per square meter (kg/m^2)	1.020×10^4
	pounds per square foot (lb/ft^2)	2,089
	pounds per square inch (lb/in.^{2} or psi)	14.50
British thermal units (Btu)	ergs	1.0550×10^{10}
	gram-calories (g-cal)	252.0
	horsepower-hours (hp-hr)	3.931×10^4
	joules (J)	1,054.8
	kilogram-calories (kg-cal)	0.2520
	kilogram-meters (kg-m)	107.5
	kilowatt-hours (kW-hr)	2.928×10^{-4}
British thermal unit per minute (Btu/min)	foot-pounds per second (ft-lb/sec)	12.96
	horsepower (hp)	2.356×10^{-2}
	watts (W)	17.57
bushels	cubic feet (ft^3)	1.2445
	cubic inches (in.^{3})	2,150.4
	cubic meters (m^3)	3.524×10^{-2}
	liters	35.24
	quarts, dry	32.0

continued

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Table A.1D.1 Unit of Measurement Conversion Chart, continued

To convert:	Into:	Use the multiplier:
degrees Celsius or Centigrade (°C)	degrees Fahrenheit (°F)	(°C × 9/5) + 32
	Kelvin (K)	(°C) + 273.15
degree Fahrenheit (°F)	degrees Celsius (°C)	5/9 × (°F – 32)
	Kelvin (K)	[5/9 × (°F – 32)] + 273.15
centimeters (cm)	feet (ft)	3.281×10^{-2}
	inches (in.)	0.3937
	kilometers (km)	10^{-5}
	meters (m)	10^{-2}
	miles	6.214×10^{-6}
	millimeters (mm)	10.0
	mils	393.7
	yards	1.094×10^{-2}
centimeters per second (cm/sec)	feet per minute (ft/min)	1.1969
	feet per second (ft/sec)	3.281×10^{-2}
	kilometers per hour (km/hr)	3.6×10^{-2}
	meters per minute (m/min)	0.6
	miles per hour (miles/hr)	2.237×10^{-2}
	miles per minute (miles/min)	3.728×10^{-4}
coulombs (C)	faradays	1.036×10^{-5}
coulombs per square centimeter (C/cm^2)	coulombs per square inch ($C/in.^2$)	64.52
	coulombs per square meter (C/m^2)	10^4
coulombs per square inch ($C/in.^2$)	coulombs per square centimeter (C/cm^2)	0.1550
	coulombs per square meter (C/m^2)	$1.55 \times \times 10^3$
cubic centimeters (cm^3)	cubic feet (ft^3)	3.531×10^{-5}
	cubic inches ($in.^3$)	6.102×10^{-2}
	cubic meters (m^3)	10^{-6}
	cubic yards	1.308×10^{-6}
	gallons, U.S. liquid	2.642×10^{-4}
	liters	10^{-3}
	pints, U.S. liquid	2.113×10^{-3}
	quarts, U.S. liquid	1.057×10^{-3}
days	hours (hr)	24.0
	minutes (min)	1.44×10^3
	seconds (sec)	8.64×10^4
degrees (of angle; °)	minutes (min)	60.0
	quadrants, of angle	1.111×10^{-2}
	radians (rad)	1.745×10^{-2}
	seconds (sec)	3.6×10^4
drams	grams (g)	1.7718
	grains	27.3437
	ounces, avoirdupois (oz)	6.25×10^{-2}
dynes (dyn)	joules per centimeter (J/cm)	10^{-7}
	joules per meter (J/m) or newtons (N)	10^{-5}
	kilograms (kg)	1.020×10^{-6}
	pounds (lb)	2.248×10^{-6}

continued

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Table A.1D.1 Unit of Measurement Conversion Chart, continued

To convert:	Into:	Use the multiplier:
faradays	ampere-hours (amp-hr)	26.80
	coulombs (C)	9.649×10^{-4}
foot-pounds per minute (ft-lb/min)	British thermal units per minute (Btu/min)	1.286×10^{-3}
	foot-pounds per second (ft-lb/sec)	1.667×10^{-2}
	horsepower (hp)	3.030×10^{-5}
	kilogram-calories per minute (kg-cal/min)	3.24×10^{-4}
	kilowatts (kW)	2.260×10^{-5}
grams (g)	decigrams (dg)	10
	dekagrams (Dg)	0.1
	dynes (dyn)	980.7
	grains	15.43
	hectograms (hg)	10^{-2}
	kilograms (kg)	10^{-3}
	micrograms (μg)	10^{6}
	milligrams (mg)	10^{3}
	ounces, avoirdupois (oz)	3.527×10^{-2}
	ounces, troy	3.215×10^{-2}
	pounds (lb)	2.205×10^{-3}
horsepower (hp)	horsepower, metric	1.014
inches (in.)	centimeters (cm)	2.540
	feet (ft)	8.333×10^{-2}
	meters (m)	2.540×10^{-2}
	miles	1.578×10^{-5}
	millimeters (mm)	25.40
	yards	2.778×10^{-2}
inches of mercury (in. Hg)	atmospheres (atm)	3.342×10^{-2}
	kilogram per square centimeter (kg/cm^2)	3.453×10^{-2}
	kilograms per square meter (kg/m^2)	345.3
	pounds per square foot (lb/ft^2)	70.73
	pounds per square inch ($lb/in.^2$ or psi)	0.4912
joules (J)	British thermal units (Btu)	9.480×10^{-4}
	ergs	10^{7}
	foot-pounds (ft-lb)	0.7376
	kilogram-calories (kg-cal)	2.389×10^{-4}
	kilogram-meters (kg-m)	0.1020
	newton-meter (N-m)	1
	watt-hours (W-hr)	2.778×10^{-4}
Kelvin (K)	degrees Celsius (°C)	K – 273.13
	degrees Fahrenheit (°F)	$[(K - 273.13) \times 9/5] + 32$
kilolines	maxwells (Mx)	10^{3}
kilometers (km)	centimeters (cm)	10^{5}
	feet (ft)	3,281
	inches (in.)	3.937×10^{4}
	meters (m)	10^{3}
	miles	0.6214
	yards	1,094.

continued

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Table A.1D.1 Unit of Measurement Conversion Chart, continued

To convert:	Into:	Use the multiplier:
kilowatts (kW)	British thermal units per minute (Btu/min)	56.92
	foot-pounds per minute (ft-lbs/min)	4.426×10^4
	horsepower (hp)	1.341
	kilogram-calories per minute (kg-cal/min)	14.34
liters	bushels, U.S. dry	2.838×10^{-2}
	cubic centimeters (cm^3)	10^3
	cubic feet (ft^3)	3.531×10^{-2}
	cubic inches ($in.^3$)	61.02
	cubic meters (m^3)	10^{-3}
	cubic yards	1.308×10^{-3}
	gallons, U.S. liquid	0.2642
	gallons, imperial	0.21997
	kiloliter (kl)	10^{-3}
	pints, U.S. liquid	2.113
	quarts, U.S. liquid	1.057
maxwells (Mx)	webers (W)	10^{-8}
micrograms (μg)	grams (g)	10^{-6}
microliters (μl)	liters	10^{-6}
milligrams (mg)	grams (g)	10^{-3}
milligrams per liter (mg/liter)	parts per million (ppm)	1.0
millihenries (mH)	henries (H)	10^{-3}
milliliters (μl)	liters	10^{-3}
millimeters (mm)	centimeters (cm))	0.1
	feet (ft)	3.281×10^{-3}
	inches (in.)	3.937×10^{-2}
	kilometers (km)	10^{-6}
	meters (m)	10^{-3}
	miles	6.214×10^{-7}
millimeters of mercury (mmHg) or torr	atmospheres (atm)	1.316×10^{-3}
	kilograms per square meter (kg/m^2)	136.0
	pounds per square foot (lb/ft^2)	27.85
	pounds per square inch ($lb/in.^2$ or psi)	0.1934
nepers (Np)	decibels (dB)	8.686
newtons (N)	dynes (dyn)	10^5
	kilograms, (force (kg)	0.10197162
	pounds, force (lb)	4.6246×10^{-2}
ohms (Ω)	megohms (MΩ)	10^{-6}
	microhms (mΩ)	10^6
ounces, avoirdupois	drams	16.0
	grains	437.5
	grams (g)	28.349527
	pounds (lb)	6.25×10^{-2}
	ounces, troy	0.9115
	tons, metric	2.835×10^{-5}

continued

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Table A.1D.1 Unit of Measurement Conversion Chart, continued

To convert:	Into:	Use the multiplier:
ounces, fluid	cubic inches (in^3)	1.805
	liters	2.957×10^{-2}
ounces, troy	grains	480.0
	grams (g)	31.103481
	ounces, avoirdupois (oz)	1.09714
	pounds, troy	8.333×10^{-2}
pascal (P)	newton per square meter (N/m^2)	1
pounds, force (lb)	newtons (N)	21.6237
pounds per square foot (lb/ft^2)	atmospheres (atm)	4.725×10^{-4}
	inches of mercury (in. Hg)	1.414×10^{-2}
	kilograms per square meter (kg/m^2)	4.882
	pounds per square inch (lb/in^2 or psi)	6.944×10^{-3}
pounds per square inch ($lb/in.^2$ or psi)	atmospheres (atm)	6.804×10^{-2}
	inches of mercury (in. Hg)	2.036
	kilograms per square meter (kg/m^2)	703.1
	pounds per square foot (lb/ft^2)	144.0
	bar	6.8966×10^{-2}
quadrants, of angle	degrees (°)	90.0
	minutes (min)	5.4×10^3
	radians (rad)	1.571
	seconds (sec)	3.24×10^5
quarts, dry	cubic inches ($in.^3$)	67.20
quarts, liquid	cubic centimeters (cm^3)	946.4
	cubic feet (ft^3)	3.342×10^{-2}
	cubic inches ($in.^3$)	57.75
	cubic meters (m^3)	9.464×10^{-4}
	cubic yards	1.238×10^{-3}
	gallons	0.25
	liters	0.9463
radians (rad)	degrees (°)	57.30
	minutes (min)	3,438
	quadrants	0.6366
	seconds (sec)	2.063×10^5
torr *see* millimeter of mercury		
watts (W)	British thermal units per hour (Btu/hr)	3.413
	British thermal units per hour (Btu/min)	5.688×10^{-2}
	ergs per second (ergs/sec)	10^7
webers (Wb)	maxwells (M)	10^8
	kilolines	10^5

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Table A.1D.2 Power of Ten Prefixes for SI Units

Prefix	Factor	Abbreviation
atto	10^{-18}	a
femto	10^{-15}	f
pico	10^{-12}	p
nano	10^{-9}	n
micro	10^{-6}	m
milli	10^{-3}	μ
centi	10^{-2}	c
deci	10^{-1}	d
deca	10^{1}	da
hecto	10^{2}	h
kilo	10^{3}	k
myria	10^{4}	my
mega	10^{6}	M
giga	10^{9}	G
tera	10^{12}	T
peta	10^{15}	P
exa	10^{18}	E

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Table A.1D.3 Celsius/Fahrenheit Temperature Conversion Chart

Degrees Celsius (°C)	Temperature	Degrees Fahrenheit (°F)
−17.8	0	32.0
−17.2	1	33.8
−16.7	2	35.6
−16.1	3	37.4
−15.6	4	39.2
−15.0	5	41.0
−14.4	6	42.8
−13.9	7	44.6
−13.3	8	46.4
−12.8	9	48.2
−12.2	10	50.0
−11.7	11	51.8
−11.1	12	53.6
−10.6	13	55.4
−10.0	14	57.2
−9.4	15	59.0
−8.9	16	60.8
−8.3	17	62.6
−7.8	18	64.4
−7.2	19	66.2
−6.7	20	68.0
−6.1	21	69.8
−5.6	22	71.6
−5.0	23	73.4
−4.4	24	75.2
−3.9	25	77.0
−3.3	26	78.8
−2.8	27	80.6
−2.2	28	82.4
−1.7	29	84.2
−1.1	30	86.0
−0.6	31	87.8
0.0	32	89.6
0.6	33	91.4
1.1	34	93.2
1.7	35	95.0
2.2	36	96.8
2.8	37	98.6
3.3	38	100.4
3.9	39	102.2
4.4	40	104.0
5.0	41	105.8
5.6	42	107.6
6.1	43	109.4
6.7	44	111.2
7.2	45	113.0
7.8	46	114.8
8.3	47	116.6
8.9	48	118.4
9.4	49	120.2
10.0	50	122.0
10.6	51	123.8

continued

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Table A.1D.3 Celsius/Fahrenheit Temperature Conversion Chart, continued

Degrees Celsius (°C)	Temperature	Degrees Fahrenheit (°F)
11.1	52	125.6
11.7	53	127.4
12.2	54	129.2
12.8	55	131.0
13.3	56	132.8
13.9	57	134.6
14.4	58	136.4
15.0	59	138.2
15.6	60	140.0
16.1	61	141.8
16.7	62	143.6
17.2	63	145.4
17.8	64	147.2
18.3	65	149.0
18.9	66	150.8
19.4	67	152.6
20.0	68	154.4
20.6	69	156.2
21.1	70	158.0
21.7	71	159.8
22.2	72	161.6
22.8	73	163.4
23.3	74	165.2
23.9	75	167.0
24.4	76	168.8
25.0	77	170.6
25.6	78	172.4
26.1	79	174.2
26.7	80	176.0
27.2	81	177.8
27.8	82	179.6
28.3	83	181.4
28.9	84	183.2
29.4	85	185.0
30.0	86	186.8
30.6	87	188.6
31.1	88	190.4
31.7	89	192.2
32.2	90	194.0
32.8	91	195.8
33.3	92	197.6
33.9	93	199.4
34.4	94	201.2
35.0	95	203.0
35.6	96	204.8
36.1	97	206.8
36.7	98	208.4
37.2	99	210.2
37.8	100	212.0

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SUPPLEMENT INDEX

How to Use This Index

A new main index is provided once a year. Thereafter, with the next three supplements, a cumulative supplement index, which amends the main index, will be provided. Thus, readers should consult both the main index and the supplement index when seeking an entry, as page numbers may change for replacement units and new entries will added.

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Measurement of Radioactivity

The SI unit for measurement of radioactivity is the Becquerel:

1 Becquerel (Bq) = 1 disintegration per second

The more commonly encountered unit is the Curie (Ci):

1 Ci = 3.7×10^{10} Bq
= 2.22×10^{12} disintegrations per minute (dpm)
1 millicurie (mCi) = 3.7×10^{7} Bq = 2.22×10^{9} dpm
1 microcurie (μCi) = 3.7×10^{4} Bq = 2.22×10^{6} dpm

Conversion factors:

1 day = 1.44×10^{3} min = 8.64×10^{4} sec
1 year = 5.26×10^{5} min = 3.16×10^{7} sec
counts per minute (cpm) = dpm/(counting efficiency)

Physical Characteristics of Commonly Used Radionuclides

Nuclide	Half-life	Emission	Range of emission, max
^{3}H	12.43 years	β	0.42 cm (air)
^{14}C	5370 years	β	21.8 cm (air)
^{32}P	14.3 days	β	610 cm (air)
			0.8 cm (water)
			0.76 cm (plexiglass)
^{35}S	87.4 days	β	24.4 cm (air)
^{125}I	60 days	γ	0.2 mm (lead)
^{131}I	8.04 days	β	165 cm (air)
		γ	2.4 cm (lead)